Genetic and functional characterisation of the autosomal dominant form of Hyper IgE Syndrome

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By

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Declaration

I, Cristina Woellner, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Background: Autosomal dominant Hyper IgE Syndrome (AD-HIES) is a rare primary immunodeficiency characterized by high serum IgE levels, eosinophilia, and skin and lung infections. Additional features of AD-HIES include characteristic facial appearance, scoliosis, retained primary teeth, and joint hyperextensibility. Recently, AD-HIES has been associated with heterozygous dominant negative mutations in the signal transducer and activator of transcription 3 (STAT3) which plays a key role in the signal transduction of a broad range of cytokines, and is crucial for IL-6-mediated regulation of Th17 cells.

Objective: We aimed to characterize patients with the clinical diagnosis of AD-HIES, to identify *STAT3* mutations, and to assess the frequency and functional consequences of these mutations. Furthermore, we studied STAT3-dependent signalling pathways in patients with an AD-HIES phenotype but no *STAT3* mutation.

Methods: We sequenced *STAT3* in 153 patients with a strong clinical suspicion of AD-HIES and further components of the IL-6 signalling pathway in patients found to be *STAT3* wild type. The impact of the mutations on immune cell function was assessed by measurement of cytokine release by immune cells, T cell phenotyping and STAT1 phosphorylation assays.

Results: About 60% of the AD-HIES patients revealed mutations in *STAT3*. All mutations found were heterozygous, clustered mainly in the DNA-binding or the SH2 domain and exerted dominant-negative effects. Functional analysis of mutations affecting different domains of STAT3 revealed that some mutations might have a less severe impact on functionality of STAT3, About 40% of our cohort of patients presenting with AD-HIES phenotype harboured wild type STAT3 and may carry mutations in other genes of either the same or closely related signalling pathways. Nevertheless, we ruled out mutations affecting the IL-6 pathway iIn five Sardinian patients with wild type STAT3.

Impact of findings: The results lead to a better characterization of heterozygous STAT3 mutations and of the pathogenesis of AD-HIES.

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Abbreviations and Symbols

AD-	Autosomal dominant
APC	Antigen-presenting cell
APS	Ammonium persulfate
AR-	Autosomal recessive
B cell	Bone marrow derived lymphocyte
BCA	Bicinchoninic acid
BCG	Bacille Calmette–Guérin
bp	base pair
BSA	Bovine serum albumin
C-	Carboxy-
CC-	Coiled-coil-
CD	Cluster of differentiaition
cDNA	complementary DNA
CMC	Chronic mucocutaneous candidiasis
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Da	Dalton
DBD	DNA-binding domain
DC	Dendritic cell
dH_2O	distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
DOCK8	Dedicator of cytokinesis 8
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
Epo	Erythropoetin
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FERM	Four-point-one, ezrin, radixin, moesin
FITC	Fluorescein isothiocyanate
GAS	Gamma activated sequence
γc	common-gamma chain
G-CSF	Granulocyte colony-stimulating factor
GEF	Guanine nucleotide exchange factors
GH	Growth hormone
GHIS	Growth hormone (GH) insensitivity syndrome
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOF	Gain of function
gp130	Glycoprotein 130
HIES	Hyper IgE Syndrome
HSCT	Haematopoietic stem cell transplantation
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IRF9	Interferon regulatory factor 9
ISGF3	IFN-stimulated gene factor 3

ISRE	Interferon stimulated response element
IU	International units
JAK	Janus kinase
JH	Janus homology
JNK	c-Jun N-terminal kinase
kb	kilobases
LIF	Leukemia inhibitory factor
LOF	Loss of function
LPS	Lipopolysaccharide
M	molar
m	mill (10^{-3})
MAPK	Mitogen-activated protein kinase
Mb	Megabase
M-CSF	Macrophage colony-stimulating factor
min	minutes
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
n	nano (10^{-9})
N-	Amino-
NIH	National Institutes of Health
NK	Natural killer cell
NKT	Natural killer T cell
nm	nanometer
NT-	Aminoterminal-
OD	Optical density
OSM	Oncostatin M
p	pico (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy7
PerCP	Peridinin-chlorophyll
pH	potentia Hydrogenii
PI3K	Phosphatidylinositide 3-kinase
PIAS	Protein inhibitors of activated STATs
PKCd	Protein kinase C delta
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonylfluorid
Prl	Prolactin
PRR	Pattern recognition receptor
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
RBC	Red Blood Cell
RNA	Ribonucleic acid
rpm	revolution per minute
RPMI	Rockwell Park Memorial Institute medium
RT	Real time
RT	Reverse transcriptase
RT	Room temperature
SAP	Shrimp alkaline phosphatase

SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulfate
SEB	Staphylococcus enterotoxin B
SH2	Src homology 2 domain
SHP	Src homology region 2 domain-containing phosphatase
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
T cell	Thymus derived lymphocyte
TAD	Transcriptional activation domain
TGF-β	Transforming growth factor β
Th	T-helper
TNF	Tumour necrosis factor
Tpo	Thrombopoietin
Tregs	regulatory T cells
TYK2	Tyrosine kinase2
µ	micro (10^{-6})
U	Units
V	Volt
w/v	weight/volume
wt	wild-type

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Chapter 1 : General Introduction

1.1 The JAK-STAT signalling pathway

Cytokines are small signalling molecules secreted by numerous cells for intercellular communication and as such they control a variety of biological responses. Each cytokine has a matching cell-surface receptor and the binding of a cytokine to its receptor induces the activation of intracellular signalling cascades leading to specific responses by the target cells. Depending on the signal, tissue, or cellular context these responses include proliferation, differentiation, migration, apoptosis, and cell survival (Alberts et al., 2002).

Members of the type I and type II cytokine receptor families have no own catalytic kinase activity and rely on the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signalling pathway (Alberts et al., 2002). Therefore, JAK-STAT signalling is essential for several developmental and homeostatic processes, such as haematopoiesis, immune cell development, stem cell maintenance, organismal growth, and mammary gland development and provides one of the most direct routes from the cell surface to the nucleus (Ghoreschi et al., 2009; Harrison, 2012).

The cytokine receptors consist of two or more transmembrane polypeptide chains. Some cytokine receptor chains are specific to a particular cytokine receptor, while others are shared among several such receptors. All cytokine receptors, however, are associated with one or more JAKs (Alberts et al., 2002).

The binding of a cytokine to its receptor induces the oligomerization of the receptor chains bringing two or more intracellular, receptor-associated JAKs into close proximity and allowing them to cross-phosphorylate each other.

Once activated, JAKs phosphorylate tyrosine residues on the cytoplasmic tail of the receptors, creating docking sites for members of the STAT family and other signalling proteins (Darnell et al., 1994; Kiu and Nicholson, 2012).

Upon binding to the receptor through their Src homology 2 (SH2) domains, the STATs become themselves phosphorylated by the JAKs. Phosphorylation of a conserved tyrosine residue located between the SH2 domain and the C-terminal transcriptional activation domain (TAD) results in the dissociation of the STAT molecules from the receptor and in the formation of phosphorylated STAT dimers (Meyer and Vinkemeier, 2004; Shuai et al., 1994).

These STAT dimers enter the nucleus where they bind to specific regulatory sequences within the promoter regions of target genes to activate or repress their transcription (Ihle, 1996; Schindler and Darnell, 1995) (Figure 1.1).

The signal transduction is terminated by dephosphorylation of STAT dimers but,contrary to the previous assumption that the dephosphorylation of the leads to the release of the STATs from the DNA, it has recently been shown that the DNA binding protects STATs from dephosphorylation (Meyer and Vinkemeier, 2004).

Once released from the DNA, STAT dimers undergo conformational changes and exit the nucleus (Mertens et al., 2006; Zhong et al., 2005).This canonical model of JAK-STAT signalling has recently been challenged by the observation that some STAT family members undergo constitutive shuttling between the nuclear and cytosolic compartments even in the absence of cytokine stimulation. Research indicated that STATs use two different import pathways: unphosphorylated STATs migrate via a carrier-free mechanism that involves direct interactions with nucleoporins, whereas the nuclear import of tyrosine-phosphorylated STAT dimers is dependent on importins (Meyer and Vinkemeier, 2004).

Both pathways operate simultaneously and make the nucleocytoplasmic cycling of STAT proteins much more dynamic than initially thought.

Based on their tissue specificity and the receptors engaged, different JAKs and STATs are recruited in the signalling event (Schindler and Plumlee, 2008).

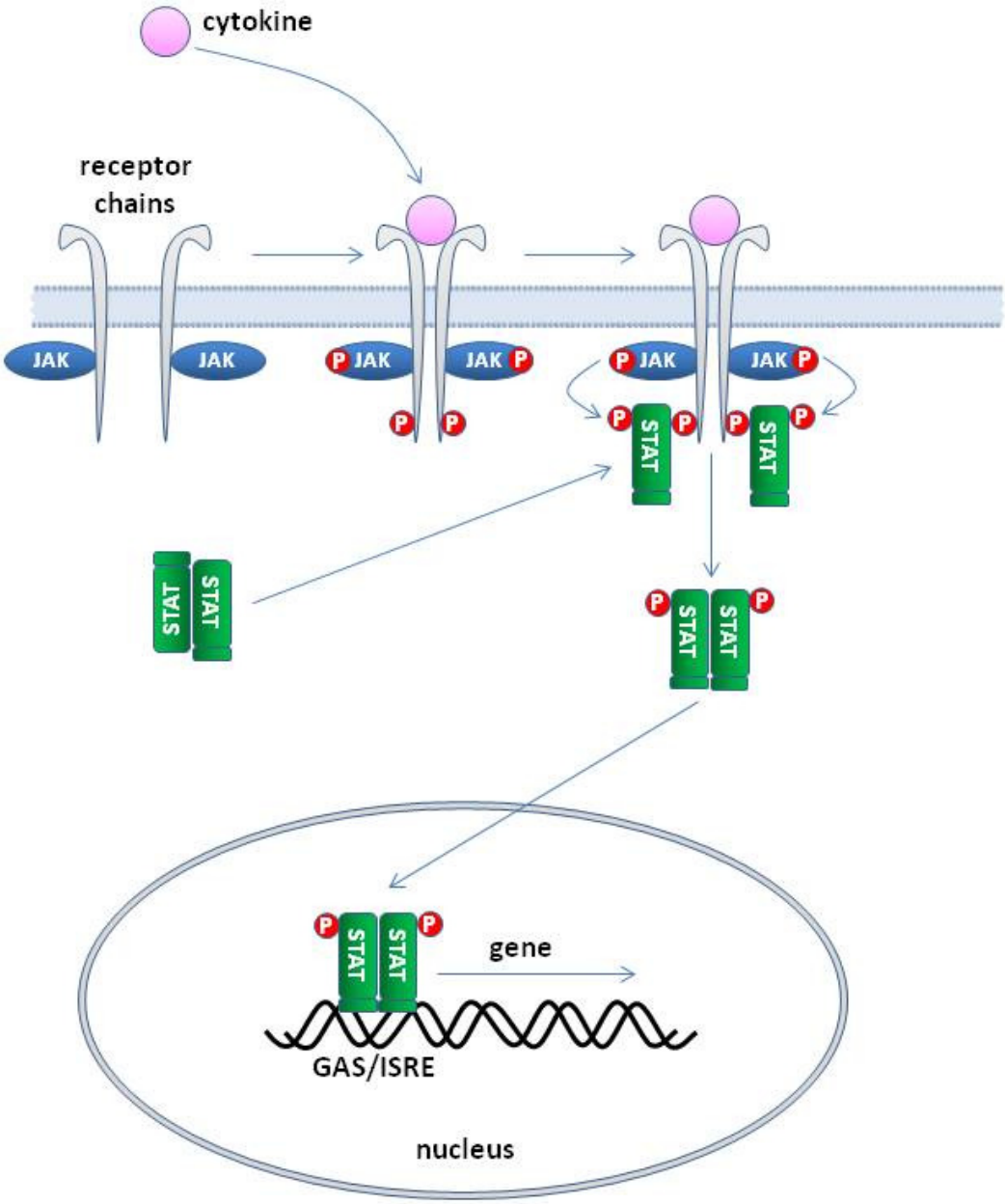


Figure 1.1: The JAK-STAT signalling pathway

Upon binding of an extracellular ligand, changes of the receptor permit the intracellular JAKs associated with the receptor chains to phosphorylate one another. Trans-phosphorylated JAKs then phosphorylate the receptor chains. STAT proteins are recruited to the resulting binding sites. Subsequently STATs get phosphorylated by the JAKs, dissociate from the receptor chains, dimerize and translocate to the nucleus where they regulate the transcription of target genes.
GAS: Interferon-gamma activated sequence; ISRE: Interferon stimulated response element

1.1.1 Janus kinases (JAKs)

Janus kinases (JAKs) are a family of intracellular, receptor associated, tyrosine kinases.

Initially JAKs were named "just another kinase". But when their importance became apparent, the acronym JAK was changed to "Janus kinase" after the 'two-faced', roman god 'Janus' in reflection of their characteristic structure (Kiu and Nicholson, 2012).

There are four known members of the mammalian JAK family, JAK1-3 and the tyrosine kinase2 (TYK2).

JAKs are large kinases with molecular weights ranging from 120-140 kDa and seven conserved regions of homology called Janus homology domains 1 to 7 (JH1-7). (JH1) at the C-terminus of the kinase is followed by a pseudokinase domain (JH2), an SH2-like domain (JH3-JH4) and finally a divergent four-point-one, ezrin, radixin, moesin (FERM) domain (JH4-JH7) at the N-terminus (Imada and Leonard, 2000; Leonard and O'Shea, 1998) (Figure 1.2).

The tyrosine kinase domain (JH1) is essential for the enzymatic activity of the JAK and contains conserved tyrosines necessary for JAK activation. The phosphorylation of the tyrosines leads to a conformational change of the protein enabling the binding of substrate. The "pseudokinase domain" (JH2), is structurally similar to a tyrosine kinase domain and negatively regulates the kinase activity of the tyrosine kinase domain JH1. The JH3-JH4 domains of JAKs share homology with SH2 domains yet lacking some of the key residues usually present within SH2 domains (Yamaoka et al., 2004).

The N-terminal FERM domain of the JAKs are involved in the binding of JAKs to the cytokine receptors (Haan et al., 2001). These domains usually form a three-lobed structure; encompassing a ubiquitin-like fold (F1), an acyl-

coenzyme A binding-like fold (F2) and a pleckstrin homology domain fold (F3) (Pearson et al., 2000).

Although once thought to reside strictly in the cytoplasm, recent evidence shows that JAK1 and JAK2 are present in the nucleus of certain cells often under conditions associated with high rates of cell growth. Therefore, besides their canonical role as activators of the STAT transcription factors, these JAKs are now known to affect gene expression by activating other transcription factors and exerting epigenetic actions by phosphorylating histone H3 (Zouein et al., 2011)

1.1.2 Signal transducers and activators of transcription (STATs)

The signal transducers and activators of transcription (STATs) are the substrates of the JAK kinases and act as transcription factors, directly linking the cytokine receptor to the regulation of gene expression. To date there are seven known members of the mammalian STAT family (STAT1, 2, 3, 4, 5a, 5b and 6 (Leonard and O'Shea, 1998). All STATs range in size from 750 to 900 amino acids and each STAT consists of seven conserved functional domains: the N-terminal (NT) domain, a coiled-coil (CC) domain, a central DNA-binding domain (DBD), a linker region, a Src-homology2 (SH2) domain followed by a single conserved tyrosine residue and a C-terminal transcriptional activation domain (TAD) (Figure 1.2) (Kiu and Nicholson, 2012; Schindler et al., 2007). The selectivity of different STATs in binding various cytokine receptors is mainly determined by differences in the SH2 domains (Murray, 2007).

In resting cells non-phosphorylated STATs form homodimers through interactions between the NT domains, and reside in the cytoplasm (Braunstein et al., 2003; Ndubuisi et al., 1999). However, upon cytokine stimulation, the STATs are recruited to the receptor where the conserved tyrosine is phosphorylated by activated JAKs. The activation of the STAT proteins in turn leads to the dissociation from the receptor chain and to the spatial reorganisation of the dimer complex, stabilized by reciprocal SH2-phosphotyrosine interactions (Kiu and Nicholson, 2012).

Phosphorylated STAT dimers translocate to the nucleus through the nuclear pore complex with a mechanism dependent on importin α-5 (Fagerlund et al., 2002; Meyer and Vinkemeier, 2004).

In the nucleus, STAT dimers oligomerize to form tetramers or higher order complexes through interactions of the NT domain. These oligomers bind to tandem sequence elements within promoter regions (often referred to as Gamma activated sequence (GAS) elements) to activate or repress the transcription of specific gene subsets (Vinkemeier et al., 1996).

Nevertheless, it has been observed that some unphosphorylated STAT family members additionally undergo constitutive shuttling between the nuclear and cytosolic compartments even in the absence of cytokine stimulation, suggesting that the nucleocytoplasmic cycling of STAT proteins is a dynamic process (Meyer and Vinkemeier, 2004)

All STATs except STAT2 can get phosphorylated on at least one serine residue in their TAD by various serine kinases (e.g. MAPK, p38, JNK, PKCδ, mTOR and PI3K) (Decker and Kovarik, 2000; Kovarik et al., 1998). This serine phosphorylation enhances the STAT-mediated gene transcription (Goh et al., 1999; Visconti et al., 2000).

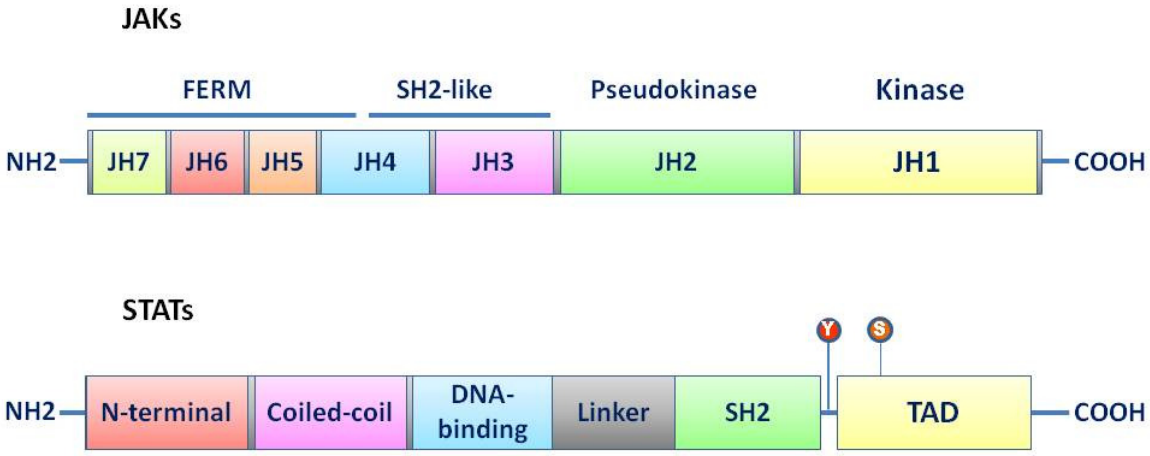


Figure 1.2: Schematic structure of JAKs and STATs

The JAK family comprises four structurally related kinases: JAK1, JAK2, JAK3, and TYK2. JAKs consist of seven JH domains. The JH1 domain is a kinase domain and the JH2 domain is a pseudokinase domain. JH3 and JH4 form the SH2-like domain, JH4-JH7 the FERM domain. The FERM domain mediates JAK binding to the transmembrane cytokine receptor.
There are seven members of the mammalian STAT family (STAT1, 2, 3, 4, 5a, 5b and 6). Each STAT consists of seven domains: the N-terminal domain, a coiled-coil domain, a central DNA-binding domain, a linker region, a SH2- domain followed by a single conserved tyrosine residue and a C-terminal transcriptional activation domain. JH, JAK homology; FERM, four-point-one, ezrin, radixin, moesin domain; SH2, Src-homology2; TAD, transcriptional activation domain.

1.1.3 Role of the JAK-STAT signalling pathway

1.1.3.1 JAKs

As most cytokine receptors do not have an own kinase activity they depend on the signal transmission by cytoplasmic, receptor-associated tyrosine kinases like JAKs (Ghoreschi et al., 2009).

JAK1, JAK2 and TYK2 are ubiquitously expressed in mammals, whereas the expression of JAK3 is restricted to cells of the hematopoietic lineage and vascular smooth muscle cells (Musso et al., 1995; Schindler et al., 2007).

Cytokines signalling via type II cytokine receptors (Type I Interferons (IFN-α/β), type II Interferon (IFN-γ), Interleukin (IL)-10, IL-20, IL-22 and IL-28), those which signal through the common γ chain (γc) (IL-2, IL-4, IL-7, IL-9, IL13, IL15 and IL-21) as well as the ones using the gp130 subunit, mainly signal through JAK1. The signal is transmitted either by JAK1 alone or in association with other JAKs (Ghoreschi et al., 2009). JAK1 knock-out mice have a severely impaired lymphopoiesis and die shortly after birth showing that JAK1 signalling is essential for the signalling of a broad range of cytokines (Rodig et al., 1998).

JAK2 is implicated in the response to hormone-like cytokines like the Growth hormone (GH), Prolactin (Prl), Erythropoetin (Epo), Thrombopoietin (Tpo), and cytokines involved in the hematopoietic cell development, such as IL-3 and Granulocyte macrophage colony-stimulating factor (GM-CSF) as well as IFN-γ (O'Shea et al., 2002; Schindler et al., 2007). Consistent with its role in erythropoiesis JAK2-deficient mice die at embryonic stages (Neubauer et al., 1998).

JAK3 exclusively mediates signals through cytokines using the common γ chain (γc) (Leonard and O'Shea, 1998; Hofmann et al., 2004). This family of cytokines comprises IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Both the γc and

JAK3 are essential for the function of all cytokine receptors of this family and are required for the development and the maintenance of the lymphoid cell system. Genetic defects of γc or JAK3 results in a severe combined immune deficiency (SCID) characterized by the lack of T, B, and NK cells in both mice and humans (Kovanen and Leonard, 2004) (see chapter 1.1.5).

TYK2, the first described member of the JAK family, associates with cytokine receptors that signal through various combinations with JAK1 and JAK2 but not with JAK3 and has major effects on the transduction of the type I IFNs, IL-6, IL-10 and IL-12/IL-23 cytokine families (Kisseleva et al., 2002) (Figure 1.3).

In addition to the cytoplasm, JAK1 and JAK2 have been found to be present as well in the nucleus where they directly affect gene expression by activating other transcription factors and exerting epigenetic actions by phosphorylating histone H3 (Zouein et al., 2011).

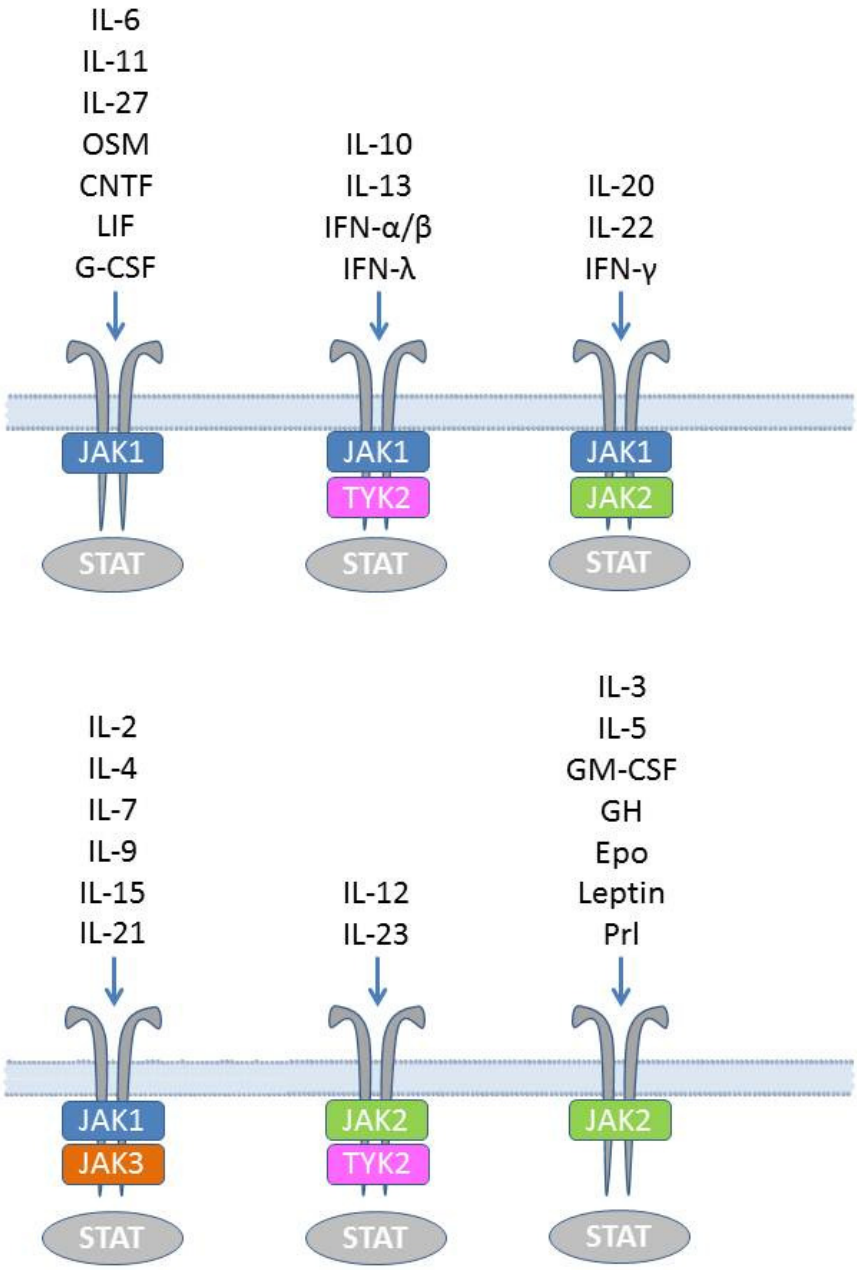


Figure 1.3: JAK signalling

Schematic representation showing the preferential cytokine/growth factor usage of different JAKs. Cytokines of the IL-6 family of cytokines as well as the Leukemia inhibitory factor (LIF) and the Granulocyte colony-stimulating factor (G-CSF) signal only via JAK1. The type I IFNs, IL-10, IL-13 and IFN-λ use a combination of JAK1 and TYK2, and IL-12 and IL-23 mediate their signals via the combination of JAK2 and TYK2. IFN-γ, IL-20 and IL-22 activate JAK1 and JAK2, whereas γc chain containing receptors, including the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, use a combination of JAK1 and JAK3. Other cytokines like IL-3, IL-5, the Granulocyte macrophage colony-stimulating factor (GM-CSF), the Growth hormone (GH), Erythropoetin (Epo), Leptin and Prolactin (Prl) signal through JAK2.

1.1.3.2 STATs

STAT1, 3, 4, 5 and 6 form homodimeric complexes, whereas STAT1 and STAT3 can, at least *in vitro*, form a heterodimeric complex (Ho and Ivashkiv, 2006). STAT2 primarily forms heterotrimers with STAT1 and the interferon regulatory factor 9 (IRF9), but can also act independently of STAT1 (Hahm et al., 2005).

STAT1 is widely expressed, with high levels in heart, thymus and spleen and has a pivotal role in the biological response to both type I and type II IFNs. Type I IFNs induces the formation of IFN-stimulated gene factor 3 (ISGF3) complexes consisting of phosphorylated STAT1, phosphorylated STAT2 and the IRF9. The complexes are assembled in the cell cytoplasm and translocated to the nucleus where they bind the interferon stimulated response element (ISRE). Stimulation with type II IFN leads to the formation of STAT1 homodimers (Durbin et al., 1996; Schindler et al., 1992; Shuai et al., 1992). In the nucleus STAT1 homodimers bind to the GAS promoter to induce expression of Interferon stimulated genes (ISG).

Like STAT3 and STAT6, STAT2 is expressed in the majority of tissues (Hou et al., 1994; Zhong et al., 1994).

STAT3 is known to transduce signals of the entire IL-6-family cytokines (IL-6, IL-11, IL-27, IL-31, the Leukemia inhibitory factor (LIF), Oncostatin M (OSM), the Ciliary neurotrophic factor (CNTF) and cardiotrophin-1), the IFN-family cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IFN-α/β and IFN-γ), the IL-2-family cytokines (IL-2, IL-7, IL-9, IL-15 and IL-21), IL-5, IL-23, the Granulocyte colony-stimulating factor (G-CSF), the Epidermal growth factor (EGF), the Macrophage colony-stimulating factor (M-CSF) and leptin (Darnell, 1997; Levy and Lee, 2002). Stat3-deficient mice die embryonically at day E6.5–7.5 underscoring the vital role of Stat3 in development (Takeda et al., 1997).

Paradoxically, STAT3 mediates signals of both pro- and anti-inflammatory cytokines; how STAT3 precisely promotes inflammation in some circumstances and inhibits it in others is unknown (El Kasmi et al., 2006; O'Shea and Plenge, 2012). Is it however known that STAT3 mediates the signals of IL-6 and IL-23 and is therefore critical for the differentiation of Th17 cells in mice and humans (Chen et al., 2006; Milner et al., 2008).

An important factor determining STAT3 functional heterogeneity is probably the existence of two alternatively splice isoforms: the fulllength STAT3α and the truncated STAT3β, which lacks the C-terminal activation domain. STAT3β was generally thought to act as a dominant negative factor but it has been shown to be involved in inflammation exerting anti-inflammatory functions by modulating directly or indirectly IL-10 expression (Maritano et al., 2004).

Unlike other STATs, such as STAT1 and STAT2, STAT3 can accumulate in the nucleus independently of its phosphorylation. The mechanisms underlying these differences relate to the involvement of distinct importins used by STATs for their nuclear import. STAT3 binds constitutively to importin-α3 and α6, and the shuttling of STAT3 in and out of the nucleus seems independent of its phosphorylation (Yang and Stark, 2008). In addition, the rate of import and export does not change following phosphorylation (Cimica et al., 2011).

It has been shown that unphosphorylated STAT3 can as well function as a transcription factor, in part by binding to unphosphorylated nuclear factor κB (NFκB) in competition with inhibitor of NFκB (IκB), driving expression of a small subset of genes that also respond to activated NFκB, (Yang and Stark, 2008; Yang et al., 2007).

The expression of STAT4 is restricted to myeloid cells, thymus and testis (Zhong et al., 1994). STAT4 is mainly phosphorylated by IL-12 and is therefore critical for the Th1 cell differentiation. STAT4 plays an analogous role in the IL-

12 dependent activation of NK cells (Kaplan et al., 1996; Thierfelder et al., 1996).

STAT5 consists of two isoforms, STAT5a and STAT5b which are closely related (96% amino acid identity) and result from a gene duplication event Both STAT5a and STAT5b exhibit differential expression in muscle, brain, mammary gland and secretary organs (seminal vesicles and salivary gland) and are required to direct the biological response to the IL-3 (IL-3, IL-5, and GM-CSF) and γc (IL-2, IL-7, IL-9, IL-15, and possibly IL-21) receptor families as well as the growth hormone (GH), prolactin (Prl), erythropoetin (Epo) and thrombopoietin (Tpo) (Liu et al., 1995, 1997; Schindler et al., 2007).

STAT6 transduces signals for both IL-4 and IL-13, which share receptor components and is therefore critical for the IL-4/IL-13-dependent polarization of naïve $CD4^+$ lymphocytes into Th2 effectors, as well as proliferation and maturation of B cells (Shimoda et al., 1996; Takeda et al., 1996) (Figure 1.4).

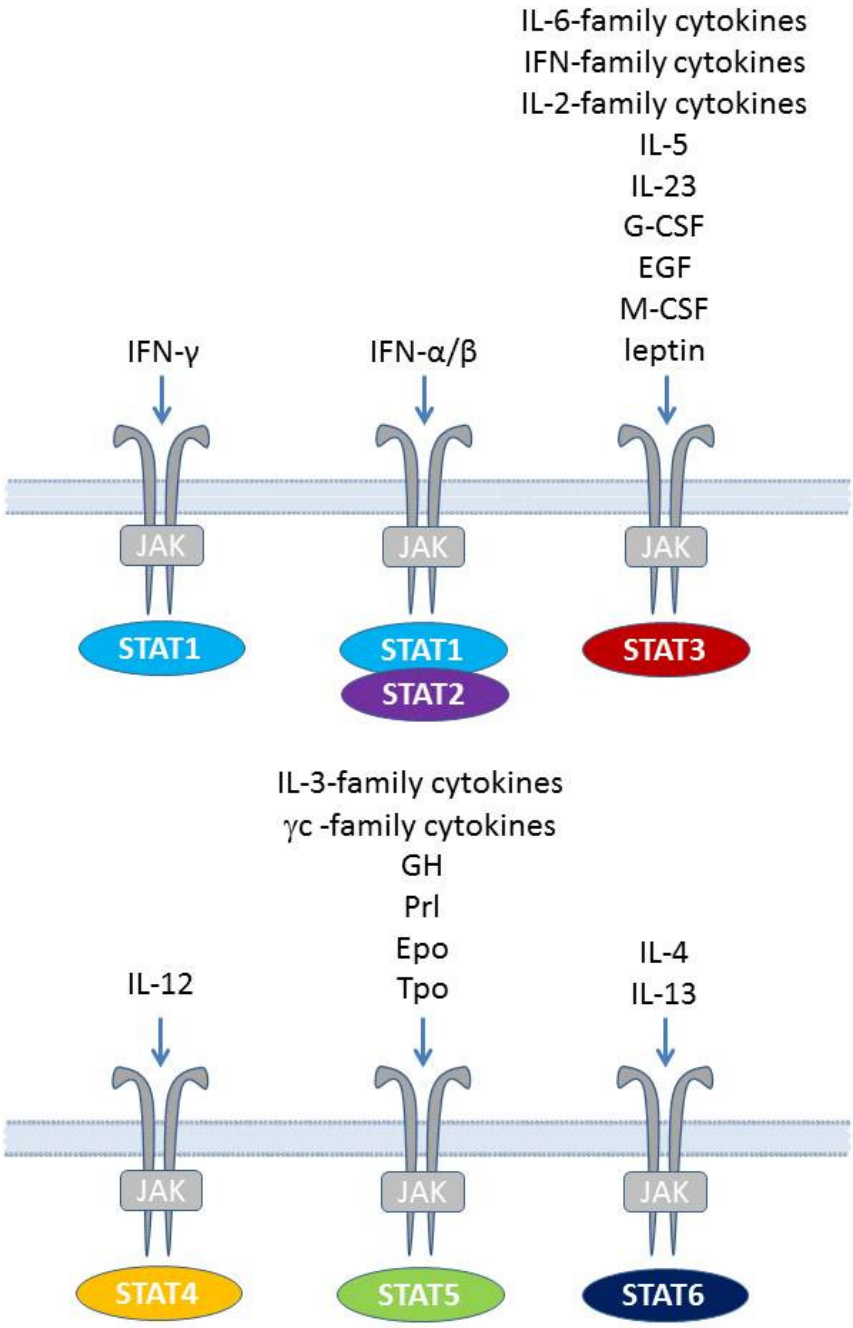


Figure 1.4: STAT signalling

Schematic representation showing the preferential cytokine/growth factor usage of different STATs. Type II IFNs signal through STAT1 whereas Type I IFNs lead to the formation of heterotrimers of STAT1, STAT2 and IRF9. STAT3 is known to transduce signals of a broad range of cytokines including the IL-6-family cytokines the IFN-family cytokines and the IL-2-family cytokines. STAT4 is mainly phosphorylated by IL-12 whereas STAT6 transduces signals for both IL-4 and IL-13. STAT5 directs the biological response to the IL-3 and γc receptor families as well as the growth hormone (GH), Prolactin (Prl), Erythropoetin (Epo) and Thrombopoietin (Tpo).

1.1.4 Regulation of the JAK-STAT signalling pathway

Regulation of the JAK-STAT pathway allows fine-tuning and prevents inappropriate cytokine responses. There are several negative feedback loops including dephosphorylation by protein tyrosine phosphatases (PTPs) and the direct inhibition by protein inhibitors of activated STATs (PIAS) and suppressor of cytokine signalling (SOCS) proteins (Kiu and Nicholson, 2012).

Several phosphatases (SHP1, SHP2, PTP 1B, TC-PTP and CD45) as well as the dual-specificity phosphatase (DUSP) family proteins have been recognized to be involved in the attenuation of the signal. These phosphatases target either the receptor complex at the membrane or the phosphorylated STAT molecules in the nucleus (Kiu and Nicholson, 2012).

The PIAS protein family members (PIAS1, PIAS3, PIASx and PIASy) inhibit STAT signalling in different ways. PIAS1 and PIAS3 bind STAT1 and STAT3 respectively and prevent them from binding DNA whereas PIASx and PIASy were shown to inhibit STAT1- and STAT4-mediated signalling without affecting the DNA-binding (Wormald and Hilton, 2004).

The family of suppressor of cytokine signalling (SOCS) proteins has eight members (SOCS1-7 and cytokine inducible SH2-containing protein CIS) and represent the best studied inhibitors of the JAK-STAT pathway. All SOCS family members have a central SH2 domain flanked by an N-terminal domain of variable length and a C-terminal domain called SOCS box (Kile et al., 2002; Starr and Hilton, 1998).

The transcription of SOCS genes is induced by activated STAT proteins creating a negative feedback loop.

Activated SOCS proteins regulate signal transduction in different ways. SOCS1 and SOCS3 can be recruited to the receptor through the SH2 domain binding either to the receptor or to the receptor associated JAKs. This way the

SOCS proteins physically obstruct the recruitment of STATs and block JAK kinase activity. In addition, SOCS proteins facilitate the ubiquitinylation of JAKs targeting them for proteosomal degradation (Wormald and Hilton, 2004).

As revealed by gene-targeting experiments, loss of SOCS1, SOCS2 or SOCS3 results in excessive STAT activity in response to IFN-γ, IL-4 (SOCS1), growth hormone (SOCS2), G-CSF and those cytokines which signal through gp130 (SOCS3) (Kimura et al., 2004; Kiu and Nicholson, 2012) (Figure 1.5).

Figure 1.5: Negative regulation of the JAK-STAT signalling pathway

The JAK-STAT signal transduction is repressed by three distinct mechanisms. SHP1 is constitutively expressed and can dephosphorylate activated JAKs or receptors. PIAS proteins are also constitutively expressed and inhibit transcriptional activation of STATs. SOCS proteins are induced in response to cytokine signalling and can inhibit JAK activity or target signalling components for ubiquitination and subsequent proteolysis. GAS: Interferon-gamma activated sequence; ISRE: Interferon stimulated response element. Figure modified after Wormald and Hilton, 2003.

1.1.5 Congenital errors of human JAK-STAT signalling

To date germline mutations in the genes encoding two human JAKs (JAK3 and TYK2) and three human STATs (STAT1, STAT3 and STAT5b) have been described. Mutations of the two *JAKs* and *STAT1* mainly manifest as immunological and infectious phenotypes, whereas STAT3 and STAT5b-deficiency comprise extrahaematological features (Casanova et al., 2012; Kralovics et al., 2005; Mead et al., 2012). In addition somatic and germline gain-of-function mutations of *JAK2* have been associated with myeloproliferative disorders (Kralovics et al., 2005; Mead et al., 2012) whereas genetic variations in components of the IL-4R axis, including STAT6, have all been associated with atopy and asthma (Chatila, 2004) (Table 1.1).

The first mutations in the human JAK-STAT pathway were characterized in the IFN-γ system; heterozygous mutations in *STAT1* were found in children highly susceptible to mycobacterial infections (Dupuis et al., 2001; Levy and Loomis, 2007). The complete deficiency of human STAT1 was described in 2003; these patients suffered from life-threatening intramacrophagic bacterial and viral diseases (Dupuis et al., 2003) but unlike patients with heterozygous *STAT1* mutations, whose outcome is favourable, these patients died in the absence of haematopoietic stem cell transplantation (HSCT) (Casanova et al., 2012). In contrast, gain-of-function mutations in human *STAT1* were found to impair IL-17 immunity and to be one cause of chronic mucocutaneous candidiasis (CMC) (Liu et al., 2011b).

JAK3 has an essential role in the signalling events of T lymphocyte cytokines; inactivating *JAK3* mutations have been documented in humans with severe combined immunodeficiency (SCID), characterised by the absence of T and NK cells, abnormal B cell function and hypoplasia of lymphoid tissues (Macchi et al., 1995; Notarangelo et al., 2000; Russell et al., 1995). The immunological

and clinical phenotype of JAK3 deficiency is indistinguishable from that of X-linked SCID. The latter syndrome is due to mutations in the interleukin-2 receptor gamma (*IL-2RG*) gene encoding the common gamma chain (γc), which is shared by cytokine receptors for interleukin IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In all these receptors the γc is coupled with JAK3, thus explaining phenotypic similarity of defects in these genes. JAK3-deficient patients present with the classical clinical features of SCID during the first few months of life, i.e. chronic diarrhoea, failure to thrive, recurrent respiratory infection and/or generalized infections from opportunistic pathogens, or signs of graft-versus-host reaction from transplacental acquired maternal T cells. The treatment of choice for JAK3 deficiency is allogeneic HSCT that has been demonstrated to be life-saving (Notarangelo et al., 2001; O'Shea et al., 2004).

As expected from the interactions of TYK2 with numerous cytokine receptors, homozygous *TYK2* mutations cause multiple defects of innate and acquired immunity (Levy and Loomis, 2007). To date two patients with TYK2 deficiency have been described. Both were highly susceptible to infections, suffered from disseminated Bacille Calmette–Guérin (BCG) and herpes zoster infections (Kilic et al., 2012; Minegishi et al., 2006).

The patient's cells showed defective responses to IL-12, type I IFNs, IL-6, IL-23 and IL-10, demonstrating the importance of TYK2 for both innate and acquired immunity in humans (Minegishi et al., 2006).

STAT5b deficiency is an autosomal recessive disorder presenting with short stature and growth hormone (GH) insensitivity syndrome (GHIS), facial dysmorphism, severe infections, and lymphoid interstitial pneumonitis (Casanova et al., 2012; Kofoed et al., 2003). STAT5b is a critical transducer of IL-2-mediated signals and is therefore essential for the development of regulatory T cells (Tregs), a subset of T cells important for the maintenance of

tolerance to self-antigens. The reduced number of Tregs associated with homozygous *STAT5b* mutations appears likely to contribute to the immunodeficiency observed in these patients (Torgerson and Ochs, 2007).

In 2007 heterozygous dominant-negative mutations in *STAT3* were shown to be causative for the autosomal dominant Hyper IgE Syndrome (AD-HIES) (Holland et al., 2007; Minegishi et al., 2007).

Gene	Type of mutation	Trait	Human Phenotype
JAK1	Not reported		
JAK2	GOF		Polycythemia vera, Hereditary Thrombocytosis, Primary myelofibrosis
JAK3	homozygous LOF	recessive	Severe combined immunodeficiency (SCID)
TYK2	homozygous LOF	recessive	Susceptibility to mycobacterial and viral infections. Elevated IgE levels in one patient
STAT1	heterozygous LOF	dominant-negative	Susceptibility to mycobacterial infections
	homozygous LOF	recessive	Susceptibility to mycobacterial and viral infections
	heterozygous GOF	dominant	Chronic mucocutaneous candidiasis (CMC)
STAT2	Not reported		
STAT3	heterozygous LOF	dominant-negative	Autosomal dominant Hyper IgE Syndrome (AD-HIES)
STAT4	Not reported		
STAT5a	Not reported		
STAT5b	homozygous LOF	recessive	Insensitivity to growth hormone Immunedysregulation due to Treg deficiency
STAT6			genetic predisposition for atopic diseases

Table 1.1: JAK-STAT mutant phenotypes in human.

LOF: loss of function, GOF: gain of function

1.2 CD4 T cell differentiation

T helper (Th) cells are an important part of the immune system, in particular of the adaptive immunity. Mature Th cells express the surface protein CD4 and are therefore usually referred to as $CD4^+$ T cells. When activated by pathogens in a specific cytokine environment naïve T helper cells differentiate into distinct lineages and acquire specialized properties and effector functions.

Initially the existence of two different effector Th cell subsets was proposed based on the cytokine profiles: Th1 and Th2 cells (Mosmann et al., 1997). Th1 cells are activated by IL-12; they produce IFN-γ and protect against intracellular pathogens. The differentiation process from naïve T cells to Th1 cells is regulated by STAT4 and the Th1 cell- lineage-restricted transcription factor T-bet. By contrast, Th2 cells differentiate in response to IL-4, which activates STAT6, resulting in the induction of GATA3. Th2 cells produce IL-4 and IL-13 and coordinate the clearance of extracellular pathogens (Bettelli et al., 2008). Recently two additional subsets of Th cells have been identified: The regulatory T cells (Tregs) which modulate the immune system, maintain tolerance to self-antigens and prevent autoimmune disease; and the Th17 cells. Th17 cells are positioned at the intersection between innate and adaptive immune response and play a significant role in defence against extracellular pathogens, as well as facultative and obligate intracellular pathogens, including intracellular bacteria and fungi (Peck and Mellins, 2010) (Figure 1.6).

1.2.1 Differentiation of Th17 cells

The differentiation of Th17 cells is orchestrated by an intricate network of signalling pathways and transcriptional regulators in T cells. Antigen-presenting cells (APCs) drive T cell differentiation by providing the antigenic, co-stimulatory and cytokine signals. Dendritic cells (DCs) are the most important APCs to bridge the crosstalk between innate and adaptive immunity (Joffre et al., 2009). Like other APCs, DCs express a repertoire of pattern recognition receptors (PRRs), that sense microbial products which are generally referred to as pathogen-associated molecular patterns (PAMPs). The binding of PAMPs to the PPRs initiates a signalling cascade culminating in the activation of DCs and the induction of adaptive immunity (Huang et al., 2012). Among the most important factors to polarize human Th17 cell differentiation is the STAT3-activating cytokine IL-6 (Laurence and O'Shea, 2007). Although the transforming growth factor β (TGF-β) plays a critical function in the differentiation of murine Th17 cells, studies indicate that this cytokine is not needed for IL-17 production in human T cells; in fact, TGF-β inhibits IL-17 production in human cells (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). Additionally, these groups showed that IL-1 is a very effective inducer of IL-17 protein expression in activated human $CD4^+$ T cells (Laurence and O'Shea, 2007).

IL-21, a cytokine produced by Th17 cells themselves, regulates the differentiation of $CD4^+$ T cells to Th17 cells in an autocrine manner, thereby amplifying the Th17 responses and inducing its own expression in an autocrine loop (Korn et al., 2007; Nurieva et al., 2007).

Finally, the heterodimeric cytokine IL-23 is important for the expansion and IL-17 production of the already Th17-committed $CD4^+$ T cells although it is

dispensable for their commitment (Stockinger and Veldhoen, 2007). Both IL-21 and IL-23 signal via STAT3 (Figure 1.6).

Essential for Th17 cell differentiation is the expression of the lineage-restricted transcription factor RORγt (Huang et al., 2012). Similar to T-bet in Th1 cells and GATA3 in Th2 cells, RORγt is highly induced in Th17 cells and plays a central role in specifying the lineage differentiation. Importantly, STAT3 deficiency greatly decreased the expression of RORγt indicating the essential function of STAT3 in the global regulation of Th17 cell gene expression and Th17 cell differentiation (Dong, 2011; Nurieva et al., 2007) (Figure 1.7).

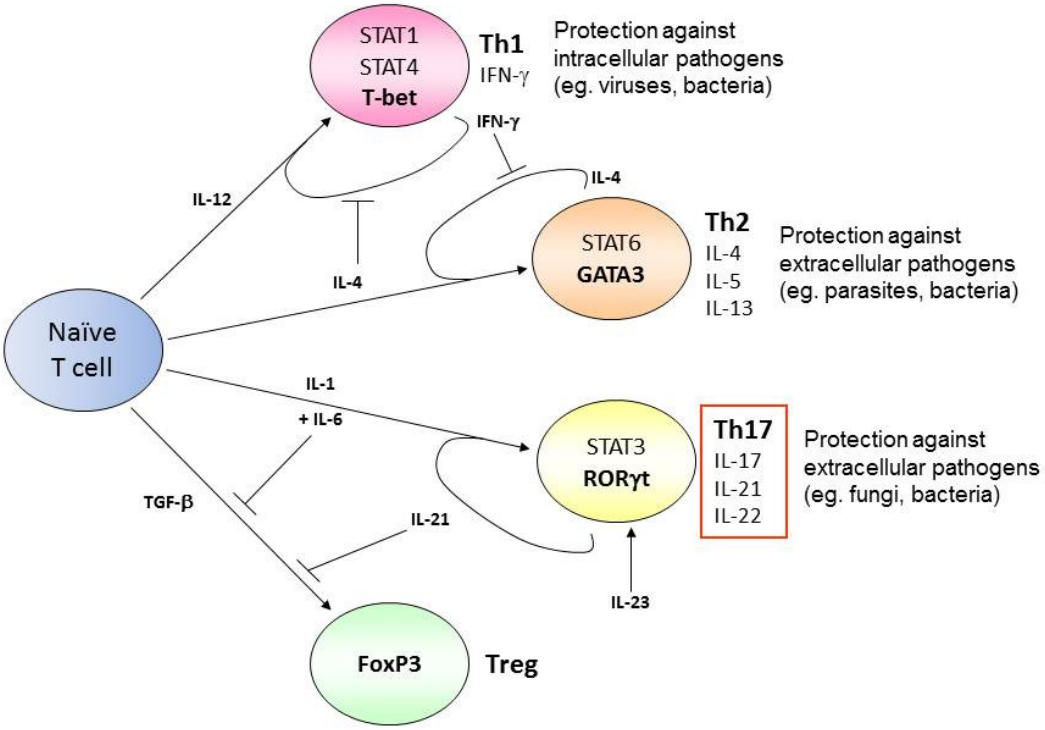


Figure 1.6: Differentiation of T helper cells

Once activated naïve $CD4^+$ T cells differentiate to become Th1 cells in the presence of IL-12. This process is regulated by STAT4 and T-bet. In response to IL-4, naïve T cells differentiate to Th2 cells through the activation of STAT6 and GATA3. In the presence of TGF-β naïve T cells become $CD4^+CD25^+Foxp3^+$ cells with suppressive effects. In the presence of IL-6, mouse naïve T cells develop to become IL-17-producing Th17 cells. In human T cells, IL-1 synergizes with IL-6 and IL-23 inducing Th17 differentiation. STAT3 and RORγt are the key transcription factors controlling this process. Figure modified after Deenick and Tangye, 2007.

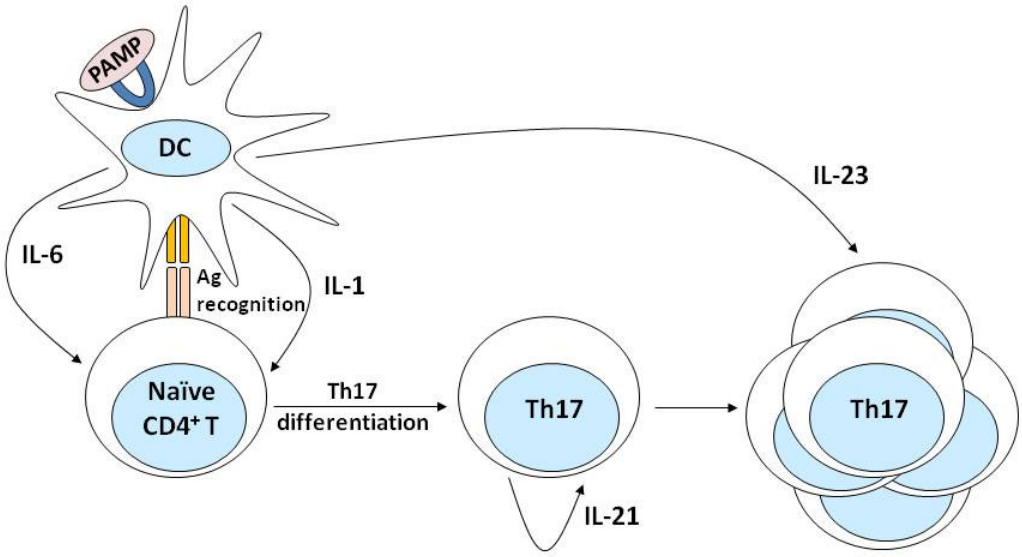


Figure 1.7: Induction of Th17 cells

The development of human Th17 cells is induced by IL-6 and IL-1 secreted by activated dendridic cells. IL-23 is not critical for Th17 commitment, but appears to be required to amplify and/or stabilize the Th17 phenotype. IL-21, a cytokine produced by Th17 cells themselves, provides an additional autocrine amplificatory signal. Figure modified after Matsuzaki and Umemura, 2007.

1.2.2 Cytokine profile and function of Th17 cells

Th17 cells are generally thought to be pro-inflammatory and play an important role in host defense against infection, by recruiting neutrophils and macrophages to infected tissues.

Th17 responses are likely to emerge as an early response to a number of pathogens not handled well by Th1- or Th2-type immunity and which require robust tissue inflammation to be cleared. Indeed, Th17 cells rapidly appear at sites of inflammation and attract other T cell subsets and neutrophils to the infection sites at later stages of the inflammatory process (Bettelli et al., 2008).

Mature Th17 cells mainly secrete IL-17A, IL-17F and IL-22 but also IL-9, IL-21, IL-26 and the chemokine CCL20. It has become clear that Th17 cells exhibit a high degree of plasticity in terms of cytokine production, which is mainly dependent on the surrounding cytokine milieu (Zhu and Paul, 2010).

IL-17A is the prototypic cytokine of the IL-17 family, which includes six members: IL-17A, B, C, D, E and F (Kolls and Lindén, 2004). IL-17A and IL-17F are both secreted by Th17 cells but also by other cell types, such as γδ T cells, NKT cells, NK cells, neutrophils, and eosinophils (Korn et al., 2009; Park et al., 2005). Both IL-17A and IL-17F induce the production of proinflammatory cytokines, chemokines and metalloproteinases from a broad range of tissues and cell types (Bettelli et al., 2008). In particular IL-17A, is involved in the recruitment, activation and migration of neutrophil granulocytes by inducing the production of colony stimulatory factors and the chemokine ligand CXCL8 by both macrophages and tissue resident cells (Annunziato and Romagnani, 2009). Thus, IL-17 is important to control and clear various pathogens including *Candida albicans* (Ouyang et al., 2008; Puel et al., 2010).

IL-22 is a cytokine of the IL-10 family of cytokines. In addition to IL-10 and IL-22 this family comprises IL-19, IL-20, IL-24, IL-26, IL-28, and IL-29. IL-22 is produced by terminally differentiated Th17 cells (Wolk et al., 2010).

Like the IL-17 receptor (IL-17R), the receptor for IL-22 (IL-22R) is widely expressed, including epithelial and endothelial cells. In contrast to IL-17R, IL-22R is not expressed on immune cells, and thus IL-22 is used by Th17 cells to communicate with tissues but not with other immune cells (Wolk et al., 2010). IL-22 induces the expression of β-defensins in epithelial cells and promotes epidermal hyperplasia suggesting an essential role for the immune barrier function of epithelial cells (Liang et al., 2006; Wolk et al., 2004; Zheng et al., 2007).

IL-21 belongs to the IL- 2 family of cytokines and uses the γc as part of its receptor. In addition to the differentiation of Th17 cells, IL-21 stimulates the proliferation and differentiation of $CD8^+$ T cells, B cell differentiation and antibody class switching and differentiation of NK cells (Korn et al., 2009).

Moreover, human Th17 cells produce the chemokine CCL20 (Wilson et al., 2007). CCL20 is a ligand for CCR6 and has antimicrobial as well as chemoattractive activity. Notably, human Th17 cells express CCR6; through CCL20 secretion, Th17 cells regulate their own recruitment to inflamed tissues (Korn et al., 2009) (Figure 1.8).

It has been shown that some T cells can produce both the hallmark Th1 cytokine IFN-γ and IL-17 in vitro and in vivo; IL-17/IFN-γ double-positive T cells are found in elevated numbers in both human- and mouse-inflamed tissues, and similar populations are observed in human Th17 cells differentiated *in vitro* from naïve $CD4^+$ T cells. Exactly where these cells fit in the spectrum of Th17 and Th1 cells is unclear as it has not been shown conclusively, whether the production of IFN-γ by Th17 cells is a protective or pathogenic mechanism

(Boniface et al., 2010; O'Shea et al., 2009). Considering the amount of tissue damage that can be induced by Th17 cells and the fact that IFN-γ-deficient animals develop exacerbated disease in several autoimmune disease animal models, it is possible that IFN-γ produced by Th17 cells ultimately serves to limit Th17-induced inflammation (Peters et al., 2011).

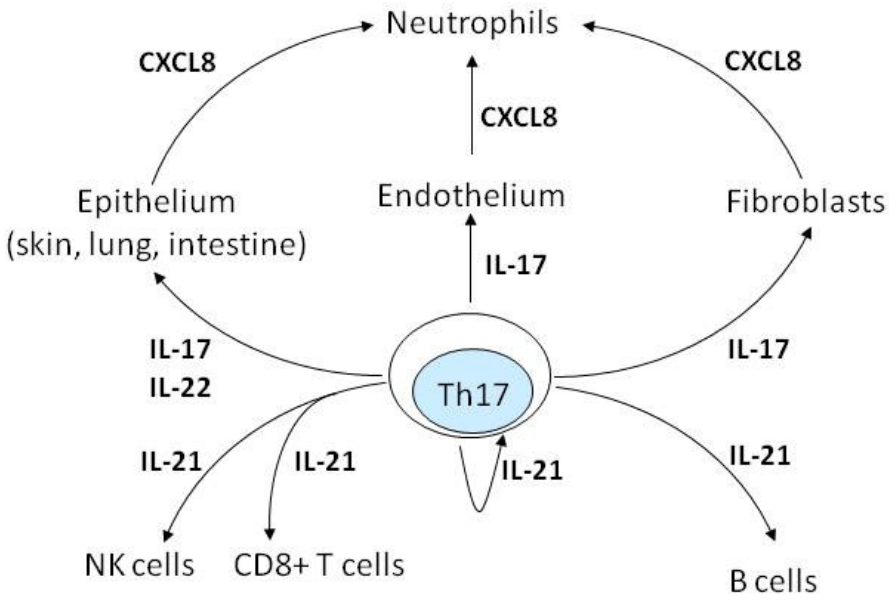


Figure 1.8: Cytokines and chemokines produced by human Th17 cells and their target

IL-17A induces epithelial cells, endothelial cells, and fibroblasts to produce the chemokine ligand CXCL8, which is crucial for the recruitment of neutrophil granulocytes. IL-22 induces the expression of β-defensins in epithelial cells. IL-21 activates NK cells and $CD8^+$ T cells and is a powerful B cell stimulatory factor. IL-21 also provides an autocrine amplification loop for Th17 cells themselves. Figure modified after Annunziato et al., 2012.

1.3 The Hyper IgE Syndrome

1.3.1 Primary immunodeficiencies –definition and classification

Primary immunodeficiencies are a heterogeneous group of disorders, which affect cellular and humoral immunity or non-specific host defense mechanisms mediated by complement proteins, and cells such as phagocytes and natural killer (NK) cells (Lim and Elenitoba-Johnson, 2004).

These disorders of the immune system cause increased susceptibility to infection, autoimmune disease, and malignancy. There are over 80 primary immunodeficiencies, many of which are very rare, and in most cases associated with inherited genetic defects (Geha et al., 2007). In the majority of cases, the primary immunodeficiencies manifest in the first year of life. They can, however, present at any age, including adulthood (Sicherer and Winkelstein, 1998). The advent of molecular genetic analyses now allows the detection and confirmation of immunodeficiencies that were otherwise not severe enough to have led to a specific diagnosis. In addition, effective treatment for many disorders has led to increased survival of many children with primary immunodeficiency into adult life (Korpi et al., 2000; Puck, 1997). The identification of many genes responsible for primary immunodeficiencies has provided insights regarding the spectrum of clinical severity seen in a particular disorder and the phenotypic overlap resulting from mutations of different genes.

Over 200 distinct genes have been identified to date, whose abnormalities account for more than 150 different forms of primary immunodeficiencies (Geha et al., 2007; Al-Herz et al., 2011; Notarangelo et al., 2009).

There are eight different classes of primary immune disorder.

1) Combined T and B cell deficiencies
2) Predominantly antibody deficiencies
3) Other well-defined immunodeficiency disorders
4) Diseases of immune dysregulation
5) Congenital defects of phagocyte number, function or both
6) Defects in innate immunity
7) Autoinflammatory disorders
8) Complement deficiencies

1.3.1.1 Epidemiology

Many immunodeficiencies are rare, however, the incidence of primary immunodeficiencies has increased by 10-fold since 1969 to 1 in 10,000. This is partly due to increased identification of affected patients, reduced morbidity and mortality from the introduction of antibiotics, enhanced methods of detection of immunological abnormalities, and the identification of gene mutations responsible for the disorders (Lim and Elenitoba-Johnson, 2004).

This figure does not take into account people with mild immune system defects who have not received a formal diagnosis.

1.3.2 **The Hyper IgE Syndrome (HIES)**

The Hyper IgE syndrome (HIES) is a rare congenital immunodeficiency (incidence <1:1 000 000), characterized by the classic clinical triad of:

- Eczema with massively elevated serum IgE (>2000 IU/mL), and eosinophilia,
- Recurrent skin abscesses, usually caused by *Staphylococcus aureus* and
- Recurrent pneumonia leading to the formation of lung cysts

The HIES usually presents within the first few years of life and has no gender predilection (Grimbacher et al., 2005).

“So went Satan forth from the presence of the Lord, and smote Job with sore boils from the sole of his foot unto his crown."

With this quote from the book of Job 2:7 Davis, Schaller and Wedgewood coined the term Job's syndrome. In 1966 they reported two girls with red hair, chronic dermatitis, and recurrent staphylococcal abscesses and pneumonias and named the disease after the biblical character Job (Davis et al., 1966). In 1972, Buckley et al. described two boys with similar symptoms who also had distinctive facial appearances and extremely elevated IgE levels (Buckley et al., 1972). Following this publication, elevated IgE were found as well in the two girls from the initial report, showing that Job syndrome and Buckley syndrome represented the same condition. This syndrome is now referred to as Hyper IgE Syndrome (HIES).

Most cases of HIES are sporadic, but both autosomal dominant (AD-HIES) and autosomal recessive traits of inheritance (AR-HIES) have been described (Grimbacher et al., 1999a; Renner et al., 2004).

1.3.3 The autosomal dominant Hyper IgE syndrome (AD-HIES)

1.3.3.1 Clinic

The autosomal dominant Hyper IgE Syndrome (AD-HIES), also named type 1 HIES has been recognized as multisystem disorder and is characterized by both immunologic and non-immunologic manifestations. High serum IgE levels, eosinophilia, eczema, skin and lung infections constitute the immunologic profile of AD-HIES, whereas characteristic facial appearance, scoliosis, retained primary teeth, joint hyperextensibility, recurrent bone fractures following minimal trauma, and craniosynostosis are the main non-immunologic manifestations (Grimbacher et al., 1999a). Main clinical symptoms of AD-HIES patients and the respective frequencies are summarized in Table 1.2.

Most sporadic patients present with AD-HIES phenotype.

	Clinical features and laboratory paramenters	Frequency
Immunologic features	Eczema	100 %
	Skin Abscesses	87%
	Recurrent Pneumonia Pneumatoceles	87 % 77%
	Mucocutaneous Candidiasis	83 %
Non immunologic features	Characteristic Face Increased nose width (interalar distance)	83 % 65 %
	Retained Primary Teeth	72 %
	Hyperextensibility	68 %
	Recurrent Fractures	57 %
	Scoliosis (more than 10°)	63 %
Laboratory Parameters	Serum-IgE (>2000 IU/mL)	97 %
	Eosinophilia	93 %

Table 1.2: Clinical features and laboratory parameters of AD-HIES patients, and their frequency.

(Grimbacher et al., 1999a).

Most of AD-HIES patients had a history of a newborn rash, which usually starts within the first weeks of life, typically presenting on the scalp and face (Eberting et al., 2004, 2004). Additionally, recurrent skin abscesses occur; these are preferably caused by *Staphylococcus aureus* and are typically 'cold' as they lack classic signs of inflammation such as redness, warmth, or pain (Chamlin et al., 2002; Minegishi and Saito, 2012).

Very often AD-HIES patients suffer from recurrent infections of the upper respiratory tract, such as sinusitis, bronchitis, otitis media and mastoiditis predominantly caused by *Staphylococcus aureus* (Grimbacher et al., 2005).

A clinical hallmark of AD-HIES is recurrent pneumonia. By early adulthood, more than 50% of patients have had three or more X-ray proven pneumonias (Grimbacher et al., 1999a). *S. aureus* is the most common pathogen, with *Streptococcus pneumoniae* and *Haemophilus influenzae* also occurring frequently (Freeman and Holland, 2010). For reasons not yet understood, tissue healing after pneumonias is abnormal and results in the destruction of lung tissue, leading to the formation of lung cavities (pneumatoceles) or scarring and thickening of the lower air ways (bronchiectasis). Once parenchymal lung damage has occurred, the colonization with opportunistic pathogens such as *Pseudomonas aeruginosa* or *Aspergillus fumigatus* is favored. These secondary infections are difficult to control and represent the major cause of morbidity and mortality for these patients (Eberting et al., 2004; Freeman et al., 2007). Additionally chronic candidiasis of the mucous membranes and/or the nail bed has been observed in more than 80% of the AD-HIES patients (Grimbacher et al., 1999a).

In addition to the accumulation of respiratory infections, other symptoms can be found in these patients: More than half of the patients suffered fractures, which often occur without adequate trauma and mainly affect the long bones

and ribs rather than the spine. Furthermore, AD-HIES is associated with a hyperextensibility of the joints, and scoliosis of varying degree and severity (Eberting et al., 2004; Grimbacher et al., 1999a). A distinct facial appearance has been described (rough skin, facial asymmetry, a prominent forehead, deep-set eyes, broad nasal bridge and a fleshy nasal tip, prognathism), along with midline anomalies (Borges et al., 1998).

Abnormalities in primary teeth exfoliation is a common finding in AD-HIES patients. Reduced resorption of the primary tooth root leads to their persistence preventing the normal eruption of permanent teeth. The absence of loss of primary teeth can cause a double row formation because the permanent teeth emerge behind the milk tooth in the dental lamina (O'Connell et al., 2000).

The role of the highly elevated polyclonal serum IgE in the pathogenesis of HIES remains unclear. Values of more than 10,000 IU/mL (raising up to 100,000 IU/mL) are characteristic for AD-HIES, however, the serum IgE levels vary widely over the time and do not correlate with the clinical presentation of the patients (Buckley and Becker, 1978). Occasionally IgE values reach normal values despite the persistence of symptoms.

Most AD-HIES patients present eosinophilia (a significantly increase proportion of eosinophilic neutrophils). However, no correlation between eosinophilia, IgE levels and infectious complications has been observed (Grimbacher et al., 1999a, 2005).

1.3.3.2 Genetics

Despite many years of intense research AD-HIES remained one of the last of the major primary immunodeficiencies for which no genetic aetiology was known.

Although Davis et al. first reported two unrelated, fair-skinned girls with red hair it soon became clear that AD-HIES can occur in all ethnic groups with approximately equal gender distribution (Erlewyn-Lajeunesse, 2000) and pedigrees of HIES families showed an autosomal inheritance (Blum et al., 1977; Donabedian and Gallin, 1983; Grimbacher et al., 1999b; Van Scoy et al., 1975).

In 1997 Hershey et al. detected a polymorphism in the IL-4 receptor (Q576R) associated with the occurrence of atopic dermatitis and suggested a link to AD-HIES. However, the correlation of the AD-HIES phenotype with the Q576R allele, was soon ruled out (Grimbacher et al., 1998; Hershey et al., 1997).

An AD-HIES patient with mental retardation who was found to have an interstitial deletion of 15–20 Mb on chromosome 4q drew attention to this region (Grimbacher et al., 1999c). Linkage analysis of 19 HIES families showed linkage to the proximal arm of chromosome 4q (Grimbacher et al., 1999b). However, six of the 19 families were not linked to this region, suggesting genetic heterogeneity (Grimbacher et al., 2005).

In early 2007 our group screened a cohort of 30 sporadic patients with the typical AD-HIES phenotype for submicroscopic insertions or deletions. Using 50k and 250k SNP mapping arrays, we were able to rule out the presence of overlapping deletions in these patients, making it unlikely that a common microdeletion, accounts for the majority of cases with AD-HIES (Pfeifer et al., 2007).

Finally in summer 2007, mutations in *STAT3* were identified as a cause of AD-HIES (Holland et al., 2007; Minegishi et al., 2007). Mutations were shown to be dominant-negative and to inhibit the activity of the wild-type allele.

1.3.3.3 Pathogenesis

Since the identification of mutations in *STAT3* as major cause of AD-HIES great efforts have been made to understand the pathogenesis of the disease that can now be explained to a certain extent.

As previously described, the STAT3 molecule plays a central role in signal transduction induced by multiple cytokines, including IL-6, IL-10, IL-11, IL-17, IL-21 and IL-22.

It has become clear that AD-HIES is a disease of both too much inflammation, as well as too little inflammation; patients lack classical inflammatory responses are often afebrile and feel well, despite serious inflammation (Freeman and Holland, 2010). This can partially be explained by the role of STAT3 in the regulation of various cytokines. In fact STAT3 is essential for the signal transduction of multiple cytokines, including the proinflammatory cytokine IL-6 as well as the anti-inflammatory cytokine IL-10 (Eberting et al., 2004). However, the precise mechanisms that allow STAT3 to promote inflammation in some instances and to inhibit inflammation at other times have not been fully resolved (O'Shea and Plenge, 2012).

Inherited deficiencies of IL-10 or IL-10 receptor (IL-10R) have been described in patients with life-threatening early-onset enterocolitis (Shah et al., 2012). The fact that the phenotype of IL-10 signalling deficiency does not overlap with to that of STAT3 deficiency may be due to the residual signalling activity of STAT3 in AD-HIES.

Mutations in *STAT3* lead to failure of Th17 cell differentiation. It has been shown that significantly reduced numbers of Th17 cells are a common feature in patients with AD-HIES. Indeed, reduced numbers of Th17 cells are thought to attribute most to the clinical phenotype of AD-HIES in particular to the high susceptibility to bacterial and fungal infections (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008).

Defective Th17 responses play a central role in several immunodeficiencies with increased susceptibility to Candida infection (Puel et al., 2012). Furthermore, IL-17 signaling is involved in neutrophil proliferation and chemotaxis. Therefore, impaired neutrophil responses and recruitment to lung and skin may, at least partly, account for the recurrent staphylococcal infections observed at these particular sites (Laan et al., 1999)

Specifically, respiratory epithelial cells and keratinocytes have been demonstrated to be tightly dependent on IL-17 for induction of antimicrobial molecules, including those responsible for antifungal responses (Minegishi et al., 2009).

Th17 cells also secrete IL-22, which is responsible for upregulating secretion of antimicrobial peptides like human beta defensins and CCL20 which have been implicated in both staphylococcal-driven atopic dermatitis and CMC (Conti et al., 2011; Kolls et al., 2008). The production of these antimicrobial peptides from keratinocytes and lung epithelial cells (when stimulated by T cells) is reduced in AD-HIES patients (Minegishi et al., 2009; Yong et al., 2012).

STAT3 has important roles in the differentiation of both osteoblasts and osteoclasts *in vitro*, and mice with Stat3-deficient osteoblasts show an osteoporotic phenotype (Itoh et al., 2006; O'Brien et al., 1999; Zhang et al., 2005). Osteoclasts from AD-HIES patients with *STAT3* mutations were shown

to have a higher bone resorption activity compared to those from control subjects. This may reflect the liability to fractures due to minor trauma observed in AD-HIES patients (Minegishi et al., 2007).

IL-11 is a member of the IL-6-type cytokine family and as such signals via STAT3. It has recently been shown that inactivation of IL-11 signalling caused by a homozygous missense mutation in the IL-11R alpha is associated with craniosynostosis and delayed tooth eruption (Nieminen et al., 2011). These missense mutations (specifically Arg296Trp) rendered IL-11R alpha unable to stimulate STAT3-mediated signal transduction, hence resulting in clinical features seen in STAT3 deficiency (Yong et al., 2012).

1.3.3.4 Diagnostic criteria

AD-HIES affects multiple organ systems and patients should be carefully evaluated.

In 1999, the National Institutes of Health (NIH) clinical HIES scoring system based on 20 clinical and laboratory findings was introduced as a diagnostic tool. A point scale was developed: more specific and objective findings were assigned more points. Scores of at least 40 points suggested AD-HIES, whereas a score below 20 made the diagnosis unlikely. For intermediate values, no firm conclusion could be reached (Figure 1.9).

This clinically validated scoring system has facilitated the clinical diagnosis of AD-HIES and should therefore preferably be used (Grimbacher et al., 1999a).

Further to this, an alternative scoring system to distinguish patients with and without *STAT3* mutations has been suggested by our group (Woellner et al., 2010).

This scoring system divided patients into three categories:

1) possibly mutant STAT3, with an IgE >1,000 IU/ml plus a weighted score of >30 of recurrent pneumonia, newborn rash, pathologic bone fractures, characteristic facies and high palate; 2) probably mutant, with these features and a lack of Th17 cells or a definite family history of HIES; and 3) definitely mutant, with these features and a dominant-negative heterozygous mutation in STAT3 (Woellner et al., 2010).

Although the scoring system represents a useful tool for screening patients for genetic testing for *STAT3* mutations, the scores should not be used to keep physicians from pursuing a molecular diagnosis in a particular patient. Patients with AD-HIES accrue findings over time. Aggressive treatment with antibiotics can forestall infectious complications that would be diagnostic if allowed to occur (Woellner et al., 2010; Yong et al., 2012).

Hyper IgE Syndrome Score

Subject:_____ DOB:_____ Scoring date:_____ Total Points:_____ Gender:

Clinical findings	Points (Circle appropriate box for each finding)									
	0	1	2	3	4	5	6	7	8	10
Highest IgE (IU/ml)	<200	200-500			501-1,000				1,001-2,000	>2,000
Skin abscesses (total #)	none		1-2		3-4				>4	
Pneumonias (X-ray proven, total #)	none		1		2		3		>3	
Parenchymal lung abnormalities	absent						bronchi ectasis		pneumatocele	
Other serious infection	none				severe					
Fatal infection	absent				present					
Highest eosinophils/ul	<700			700-800			>800			
Newborn rash	absent				present					
Eczema (worst stage)	absent	mild	moderate		severe					
Sinusitis, otitis (# times in worst year)	1-2	3	4-6		>6					
Candidiasis	none	oral, vaginal	fingernail		systemic					
Retained primary teeth	none	1	2		3				>3	
Scoliosis, max curve	<10°		10-14°		15-20°				>20°	
Fractures with little trauma	none				1-2				>2	
Hyperextensibility	absent				present					
Characteristic face	absent		mild			present				
Increased nose width (interalar distance)	<1 SD	1-2 SD		>2 SD						
High palate	absent		present							
Midline anomaly	absent					present				
Lymphoma	absent				present					

Figure 1.9: Clinical HIES scoring system based on 20 clinical and laboratory findings.

(Grimbacher et al., 1999b)

1.3.4 The autosomal recessive Hyper IgE syndrome (AR-HIES)

The autosomal recessive form of HIES (AR-HIES), or type 2 HIES is very rare. Like the more frequent AD-HIES the disorder is characterized by the triad of highly elevated levels of IgE in serum, recurring abscesses of the skin, and recurrent pneumonia (Grimbacher et al., 2005; Renner et al., 2004).

In contrast to AD-HIES, the AR variant is further characterized by a high susceptibility to viral infections such as herpes simplex and herpes zoster, molluscum contagiosum and human papillomavirus. These infections are usually extensive, difficult to control and mutilating and often occur concurrently (Su et al., 2011).

Other clinical features such as the involvement of the CNS (facial paralysis, hemiplegia, ischemic infarction, and subarachnoid hemorrhage), or autoimmune phenomena are variably associated with the disease whereas vascular disorders appear to be common. The AR-HIES has high mortality due to sepsis and early onset of malignancies. Unlike AD-HIES, 50–80% of patients develop severe allergies, including anaphylaxis, to food and environmental antigens, as well as asthma (Su, 2010). The dental, skeletal and connective tissue anomalies, as well as the characteristic facies and pneumatoceles present in AD-HIES are absent in AR-HIES (Renner et al., 2004).

In all patients with AR-HIES both highly elevated serum IgE levels and increased IgG levels could be detected. Although eosinophilia is a common finding in both AD- and AR-HIES it tends to be more severe in the AR variant and often reaches values of up to 17.500/µL (normal range <700/µL) (Renner et al., 2004).

1.3.4.1 Genetics and pathogenesis

The genetic defect underlying AR-HIES has only recently been unveiled.

In 2006 a single patient with AR-HIES-like phenotype was reported with a homozygous deletion of four base pairs in the TYK2 gene leading to absence of TYK2 protein (Minegishi et al., 2006).

The patient described was a 22 year old male from a consanguineous marriage, suffering from eczema, recurrent sinopulmonary infections, candidiasis Molluscum contagiosum and herpes infections with high serum IgE levels.

However, unlike the other AR-HIES patients, he had experienced an episode of Bacille Calmette-Guerin (BCG) infection and non-typhi salmonella gastroenteritis. Subsequently, our group showed that TYK2 deficiency is not a common cause for the AR-HIES. We concluded that TYK2 deficiency more likely represents a distinct, yet similar, immunodeficiency disorder (Woellner et al., 2007).

In 2012, a second case of TYK2 deficiency was published. This patient suffered from disseminated BCG infection, neurobrucellosis, and cutaneous herpes zoster infection but did not have a hyper IgE phenotype (Kilic et al., 2012).

Homozygous mutations of the dedicator of cytokinesis 8 (*DOCK8*) gene have been shown to be responsible for many, although not all, cases of autosomal-recessive Hyper IgE syndrome (Engelhardt et al., 2009; Zhang et al., 2009). Since the discovery in 2009 that loss-of-function mutations in *DOCK8* underlie AR-HIES, an estimated more than 100 patients worldwide have been identified.

DOCK8 is a member of the DOCK family of guanine nucleotide exchange factors (GEFs), which function as activators of small G proteins (such as RAC

and CDC42) and play roles in regulation of cell migration, morphology, adhesion, and growth (Ruusala and Aspenström, 2004). DOCK8 is highly expressed within the immune system, especially in lymphocytes, suggesting crucial functions in these cell types and DOCK8 deficiency appears to impair the $CD4^+$ and $CD8^+$ T cell proliferative responses (Lambe et al., 2011).

The mutations found appear unique to each family. They encompass large deletions, point mutations that alter splicing to cause nonsense mutations, in-frame nonsense mutations, and small insertions and deletions that cause out-of-frame nonsense mutations (Engelhardt et al., 2009).

1.3.5 **Management, treatment and prognosis of HIES**

The therapeutic approach involves prevention and management of infections. Long-term administration of systemic antibiotics, antifungal and antiviral drugs is recommended. Lung abscesses may require surgery but possible complications require close attention (Freeman and Holland, 2010). For both, STAT3 and DOCK8 deficiency haematopoietic stem cell transplantation (HSCT) has been shown to be curative (Barlogis et al., 2011; Goussetis et al., 2010; McDonald et al., 2010). However, due to several side effects including massive immunosuppression, HSCT is a risky intervention and should be considered only in severe cases.

Although AD-HIES is associated with significant morbidity and mortality, adequate care, close monitoring and patient compliance has improved the prognosis, with survival to 50 years of age or over. For DOCK8-deficient AR-HIES the prognosis without HSCT is poor.

1.4 Aim of the thesis

Despite many years of intense research, the genetic aetiology of AD-HIES remained elusive until 2007, when heterozygous mutations in *STAT3* were identified as a cause of AD-HIES.

The aims of this thesis were:

- to characterize a cohort of 153 patients with the clinical phenotype of AD-HIES, to identify *STAT3* mutations and to assess their frequency
- to explore the functional consequences of various *STAT3* mutations and to associate the kind of mutation with the patients' phenotype
- to assess whether the application of functional tests and the enumeration of IL-17 producing T-helper cells might be used to predict the presence of a STAT3 mutation, therefore avoiding to sequence the entire STAT3 gene.
- to further characterize HIES patients without *STAT3* mutations by sequencing genes of related signalling pathways and analyzing cytokine release profiles of immune cells after stimulation.

Studies of patients with AD-HIES can be used to explain the role of Th17 cells in infection and in other forms of dysregulated immunity.

In general, the proposed work aimed at generating basic knowledge of the molecular biology of STAT3 signalling in AD-HIES patients and thus may pave the way to develop alternative therapies for patients suffering from either AD-HIES or other diseases presenting with imbalanced Th17 cells.

Chapter 2 : **Material and Methods**

2.1 Patients and Controls

In a worldwide collaboration we have collected genomic DNA or RNA of 153 unrelated patients with the suspected diagnosis of autosomal dominant Hyper IgE Syndrome (AD-HIES); 125 patients were referred to us with a completed NIH-clinical scoring sheet, of the remaining 28 patients DNA or blood was sent without detailed clinical information (but according to the referring immunologist all with strong suspicion of AD-HIES). Nine patients had one or more affected family members (Figure 3.14).

Of the 153 patients 80 were male and 73 female; the age of the patients at the time of clinical evaluation ranged between 1 and 59 years. One hundred and twenty-four patients came from Europe (44 from Great Britain, 25 from Italy, 18 from Poland, 14 from Germany, 10 from Spain, 3 from Sweden, 2 from Ireland, 2 from Greece, 2 from Finland, 2 from the Czech Republic, one form Norway and one from Austria), 21 from the Middle East (10 from Iran, 6 from Israel and 5 from Turkey), 7 from South America (Colombia), and 1 from North America (Canada).

Ninety-two of the 125 patients who were referred to us with clinical information, had HIES scores ≥40, suggesting that these patients probably had AD-HIES, whereas 33 of the 125 patients had scores below 40, suggesting a diagnostic uncertainty or a variant of AD-HIES. Detailed information on patients, including available clinical scores and detected *STAT3* mutations, are summarized in Table 1 of the Appendix.

Genomic DNA from 100 healthy Caucasian subjects were used as control. In addition, 24 controls were studied for their lymphocyte phenotype. All patients and controls or their parental or legal guardians provided written consent for the study in line with local ethics committee requirements.

2.2 Molecular Biology

2.2.1 Isolation of leukocytes from whole blood

Leukocytes from patients and healthy controls were extracted from 5-10mL whole blood. 30mL of RBC (Red Blood Cell Lysis Buffer; 10mM NH_4HCO_3 and 0.14M NH_4Cl) buffer were added to 10mL whole blood, mixed by inverting several times and incubated on ice for 10 minutes. After centrifugation (10 min at 3500rpm) the supernatant was carefully discarded, leaving some residual liquid and the white blood cell pellet. The pellet was re-suspended in the residual liquid and the tube vortex vigorously. To eliminate residual erythrocytes the procedure was repeated adding 20mL of RBC buffer to the re-suspended cell pellet. Isolated leukocytes were then subjected to DNA or RNA extraction.

2.2.2 DNA isolation

DNA of the patients and the healthy controls was isolated either from purified Leukocytes obtained as described above or from Peripheral Blood Mononuclear Cells (PBMCs) isolated as described in section 2.4.1.

For the isolation of the DNA the Gentra® Puregene® Blood Kit (Qiagen, UK) was used according to the instructions of the manufacturer.

In brief: 5mL of cell lysis solution (Gentra® Puregene®, Qiagen, UK) were added to pelleted leukocytes or PBMCs and mixed thoroughly to lysate the cells. Subsequently 3mL of protein precipitation solution (Gentra® Puregene® Qiagen, UK) were added to the cell lysate. The sample was then centrifuged at 3500rpm for 20 minutes at 4℃. After centrifugation 10mL isopropanol were added into a clean 50mL tube and the supernatant from the previous step was added carefully. The tube was then inverted gently 50 times until the DNA

became visible as threads or clumps. The precipitated DNA was centrifuged at 3500rpm for 10 minutes at 4°C, the supernatant was discarded and the pellet washed with 70% ethanol and centrifuged as above. After discarding the supernatant, the pellet was dried at room temperature for approximately 1 hour and rehydrated in appropriate amount of DNA hydration solution (Gentra® Puregene®, Qiagen, UK) depending on pellet size.

2.2.3 RNA isolation

Total RNA was isolated either from purified leucocytes or from peripheral blood mononuclear cells (PBMCs) using an RNA Isolation kit (RNeasy® Mini Kit, Qiagen, UK) according to manufacturer's instructions.

2.2.4 Determination of DNA and RNA concentration

To determine the DNA or RNA concentration samples were diluted 1:50 or 1:100 with ddH_2O. The absorbance of the sample was then measured photometrically of 260nm. To assess the purity of the nucleic acid the absorption of proteins was measured in parallel at a wavelength of 280nm. Ratio of readings at 260nm and 280nm [A260/A280] provides an estimate of purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 – 2.0. If there is significant contamination with protein the ratio will be lower and accurate quantitation is not possible.

2.2.5 cDNA synthesis (Reverse Transcription)

For the synthesis of complementary DNA (cDNA) from total RNA the QuantiTect® Reverse Transcription Kit (Qiagen, UK) was used according to the

instructions of the manufacturer. The amount of template RNA used for reactions ranged between 200-500ng.

2.2.6 **Polymerase chain reaction (PCR)**

Genomic DNA or cDNA was amplified by polymerase chain reaction (PCR) in 20µl reactions. All PCR consumables were purchased from Qiagen (UK) or PeqLab (UK).

Standard 20µl PCR reactions were pipetted as follows:

- 2µL 10x PCR-reaction-buffer
- 2µL 1mM dNTP-Mix
- 0,5µL 25µM *forward*-Primer
- 0,5µL 25µM *reverse*-Primer
- 0,2µL *Taq* DNA Polymerase (1U)
- 1µL Template-DNA (ca. 50ng)
- 4µL 5x Q-Soultion or enhancer solution (if needed)
- ddH_2O

Total volume 20µl

The following PCR program was used:

	Temperature in °C	Time	Number of Cycles
Denaturation	95	5 min	1
Denaturation Annealing Extension	95 50-60 72	15 sec 30 sec 50 sec - 3 min	30
Extension	72	10 min	1

The annealing temperature used was determined either by the 4GC/2AT-rule or by a gradient PCR. The extension times were chosen according to the expected PCR product lengths (approximately 60 sec extension time per 1 kb).

Primers used for the amplification are listed in the Appendix.

2.2.7 Analysis of PCR products

All amplified PCR products were analyzed by horizontal gel electrophoresis in 1.0% agarose gels. 0.5µg/mL ethidium bromide (Sigma-Aldrich, UK) were added to the gel to enable the detection of double stranded DNA fragments with ultraviolet light. Two µL PCR product were diluted with 6x loading buffer (Sigma-Aldrich, UK), loaded on the gel and separated for 30 minutes at 100V. Afterwards the DNA fragments were visualized with a gel documentation system with UV light (295 nm). The correct PCR product size was verified using the molecular weight marker Hyperladder I (200bp- 10.000bp) (Bioline, UK).

2.2.8 Purification of PCR products

For removal of unincorporated dNTP's and excess primers interfering with the subsequent sequencing, PCR reactions were digested with 1µL Shrimp alkaline phosphatase (SAP; Promega, UK) and 0.5µL Exonuclease I (Thermo Scientific, UK) which were added to the residual 18µL PCR reaction. SAP catalyzes the dephosphorylation of 5' phosphates from nucleic acids and is completely and irreversibly inactivated by heat treatment for 15 minutes at 65°C whereas the Exonuclease catalyzes the removal of nucleotides from single-stranded DNA in the 3' to 5' direction. The enzyme digestion was

performed in a PCR machine, for 15 minutes at 37°C followed by an enzyme inactivation step of 15 minutes at 80°C.

2.2.9 **Sequencing reaction**

Purified PCR products were sequenced with ABI PRISM BigDye Terminator cycle ready reaction kit V3.1 (Applied Biosystems).

Sequencing reactions were pipetted as follows:

- 1 - 3µL PCR-product (~ 50 ng)
- 1µL Big Dye Terminator 3.1
- 1,3µL Primer (2,5 µM)
- ddH_2O

Total volume 20µl

For the sequencing-reaction the following PCR program was used:

	Temperature in °C	Time	Number of Cycles
Denaturation	96	1 min	1
Denaturation Annealing Extension	96 50-60 60	10 sec 5 sec 4 min	30

Annealing temperatures and primer sequences used were the same as for the previous PCR reactions.

To remove unincorporated dye terminators and excessive primers, the sequencing reaction products were precipitated.

Ethanol precipitation is a commonly used technique for concentrating and de-salting nucleic acid (DNA or RNA) preparations in aqueous solution. The basic

procedure is that salt and ethanol are added to the aqueous solution, which forces the nucleic acid to precipitate out of solution. The precipitated nucleic acid can then be separated from the rest by centrifugation.

Two µL 125mM EDTA, 2µL 3M sodium acetate and 50µL 100% ethanol were added to the sequencing reactions. Samples were incubated at room temperature for 15 minutes in the dark and then centrifuged at 3500rpm for 30 minutes at 4°C. The supernatant was discarded carefully without disrupting the pellet. The pellet was washed with 120µL 70% ethanol and again centrifuged (3500rpm, 10 min, 4°C). The supernatant was discarded and the pellet dried at 96°C for 3 minutes to eliminate the residual ethanol. The pellet was then re-dissolved in 50µL H_2O before loading onto the sequencer.

Sequencing was carried out with the 3130xl Applied Biosystems Genetic Analyzer.

2.2.10 Analysis of sequences

Data obtained were analyzed with Sequencing Analysis software, version 5.2 (Applied Biosystems) and Sequencher™, version 4.8 (Gene Codes Corporation). Reference sequences were available from the genbank "Ensemble" (http://www.ensembl.org/index.html).

2.2.11 Bioinformatic tools

2.2.11.1 PolyPhen-2

PolyPhen-2 (Polymorphism Phenotyping v2) is a web-based tool, which predicts the possible impact of an amino acid substitution on the structure and function of a human protein using physical and comparative considerations.

For each amino acid substitution both a qualitative prediction (one of 'probably damaging', 'possibly damaging', 'benign' or 'unknown') and a score is given. The PolyPhen score represents the probability that a substitution is damaging (http://genetics.bwh.harvard.edu/pph2),

2.2.11.2 Mutation Taster

MutationTaster is a free, web-based application for rapid evaluation of the disease-causing potential of DNA sequence alterations. MutationTaster integrates information from different biomedical databases and uses established analysis tools. Analyses comprise evolutionary conservation, splice-site changes, loss of protein features and changes that might affect the amount of mRNA. Test results are evaluated by a naive Bayes classifier, which predicts the disease potential (http://www.mutationtaster.org and Schwarz et al., 2010).

2.3 Protein Biochemistry

2.3.1 Preparation of protein extracts

Cell extracts of patients' and healthy donors' PBMCs were prepared by using cell lysis buffer (Cell Signaling, UK) supplemented with 1mM of the protease inhibitor Phenylmethylsulfonylfluorid (PMSF). 10^7 cells were first washed once with PBS and pelleted by centrifugation before 400µl of lysis buffer were added. Cells were incubated on ice for 15 minutes and sonicated briefly. Afterwards extracts were centrifuged at 14,000g for 10 minutes at 4°C. Protein concentration of the supernatants was measured and extracts were stored at -80°C until further use.

2.3.2 Determination of protein concentration

The protein content of cell lysates was determined using the BCA™ protein assay kit (Thermo Scientific, UK).

With this kit the total protein concentration is exhibited by a colour change of the sample solution from green to purple in proportion to protein concentration. The colour change can then be measured using a photometer.

In a first step the proteins and Cu^{2+} ions form a Cu^{2+}-protein complex under alkaline conditions which is followed by the reduction of the Cu^{2+} to Cu^{1+}. The amount of reduced Cu^{2+} is proportional to the amount of protein present in the solution. Next, bicinchoninic acid (BCA) forms purple-blue complexes with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+}. The purple-coloured reaction products strongly absorb light at a wavelength of 562 nm. The absorbance is directly proportional to protein concentration.

A calibration curve for the quantification of protein content was created using serial dilutions of Bovine serum albumin (BSA).

2.3.3 SDS-Polyacrylamide-gelelectrophoresis (SDS-PAGE)

Proteins of cell extracts were separated according to their molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS is an anionic detergent which denatures proteins and confers negative charge to the polypeptide according to its length. To reduce disulphide bridges in proteins 2- mercaptoethanol was added. In these denaturing SDS-PAGE separations therefore, migration through polyacrylamide gel is determined only by molecular weight.

The most commonly used system of SDS-PAGE is the Laemmli method.

The Laemmli system uses buffers of different pH and composition to generate a voltage gradient and a discontinuous pH between a stacking and a resolving gel. A lower concentrated stacking gel (pH 6.8) is poured on top of a more concentrated resolving gel (pH 8.8). The stacking gel concentrates the proteins on top of the resolving gel. After entering the resolving gel the proteins are separated according to relative molecular size.

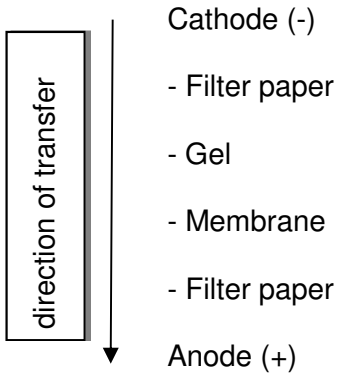
For our purpose a stacking gel with an acrylamide concentration of 6% (0.5 Tris-HCl pH 6.8; 20%(w/v) SDS; Acrylamide/Bis-acrylamide (30%/0.8% w/v); 10%(w/v) ammonium persulfate (APS); 0,02mL TEMED) and a resolving gel with a concentration of 10% was used (1.5M Tris-HCl pH 8.8; 20%(w/v) SDS; Acrylamide/Bis-acrylamide (30%/0.8% w/v); 10%(w/v) ammonium persulfate (APS); 0,02mL TEMED).

The protein extracts to analyse (5-20µg total protein) were added to 5x sample buffer (10%(w/v) SDS; 10mM beta-mercapto-ethanol; 20%(w/v) Glycerol; 0.2M Tris-HCl pH6.8; 0.05%(w/v) Bromophenolblue), denatured for 5 minutes at

95°C, cooled on ice and applied to the gel. The electrophoresis was carried out for about 2 h at 120V in SDS-PAGE running buffer (25mM Tris-HCl; 200mM Glycine; 0.1%(w/v) SDS). The size of the proteins was assessed by adding a size standard.

2.3.4 **Transfer of proteins separated by gelelectrophoresis on membranes (Western Blot)**

In order to make them accessible to antibody detection, proteins separated by gel electrophoresis were moved from within the gel onto a polyvinylidene difluoride (PVDF) membrane by a second electrophoretic transfer by using a semi-dry transfer system. The gel and membrane were sandwiched between two stacks of filter paper wetted with transfer buffer (25mM Tris-HCl; 250mM Glycine; 20% methanol) and placed in a transfer chamber as shown below.



Application of voltage across the electrodes makes the proteins migrate from the gel to the membrane. The transfer took an hour at a voltage of 12V.

2.3.5 Detection of electrophoretically separated proteins with specific antibodies

After the transfer the membrane was incubated for one hour with 5% (w/v) milk powder in PBS +0.1%Tween20 to block unoccupied binding sites on the membrane and thus to prevent nonspecific binding of antibodies. After this blocking step the membrane was incubated overnight at 4°C with the primary antibody (diluted according to manufacturer's recommendations in PBS-Tween containing 5% milk powder).

After three washing steps with PBS-Tween (15 min each), the membrane was incubated for 1 hour at RT with a horseradish peroxidase-linked secondary antibody. After another washing step the secondary antibodies were visualized using a chemiluminescence-based kit (LumiGLO®, Cell Signalling, UK).

The chemiluminescent reaction is based on the emission of light during the horse radish peroxide-catalyzed oxidation of luminol. The kit was used according to the instructions of the manufacturer. After incubation the excess of LumiGLO® was drained, the membrane wrapped in plastic and exposed to an X-ray film.

2.3.6 TNF-α Enzyme linked immunosorbent assay (ELISA)

To assess the capability of IL-10 to inhibit the TNF-α release in patients'/healthy controls' PBMCs and/or monocytes/macrophages, these cells were pre-incubated with 25ng/mL IL-10 (R&D, UK) for 1 hour and then stimulated overnight with 50ng/mL *Escherichia coli* LPS (Sigma, UK). TNF-α release was measured by using an ELISA self-development kit (PeproTech, UK) in accordance with the manufacturer's instructions. In brief, ELISA-microplates (Nunc, Germany) were coated overnight with anti-TNF-capture antibodies. After three washing steps with washing buffer (0.05% Tween-20 in

PBS), 300µL blocking buffer (1% BSA in PBS) were added to each well and incubated for 1 hour to prevent unspecific bindings. After a further washing step, samples and serial dilutions of a TNF-α standard provided by the manufacturer were added to the wells in duplicates and incubated for 2 hours. The plate was then washed and 100µl biotinylated detection antibody were added to the wells containing standards/samples. After another 2-hour incubation and a further washing step, Avidin-HRP conjugate were added for 30 minutes. Finally, 100µl substrate 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Liquid Substrate (Sigma-Aldrich, UK) were added to each well. The development of a greenish color indicated the presence of TNF-α in the samples/standards. Quantitative analysis was carried out by using an ELISA reader; optical densities were read at 405nm with wavelength correction set at 650nm. Accurate TNF-α concentrations were worked out using the Magellan software (Tecan, Switzerland).

2.4 Cellular Biology

2.4.1 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood or buffy coats.

EDTA-blood of patients was sent by referring physicians, blood of healthy controls was obtained from volunteer donors from the National Blood Service. PBMCs were separated from whole blood by gradient separation using Lymphoprep® (Axis-Shield, UK). The blood was mixed 1:1 with PBS, gently layered onto 15mL of Lymphoprep® in 50mL tubes and centrifuged at 1600rpm for 20 minutes. Mononuclear cells formed a distinct band at the sample interface between plasma and Lymphoprep® while red cells were pelleted at the bottom of the tube (Figure 2.1). PBMCs were harvested with a Pasteur pipette, washed with PBS and pelleted by centrifugation for 5 minutes at 1500rpm. Cells were then processed immediately or frozen for later use.

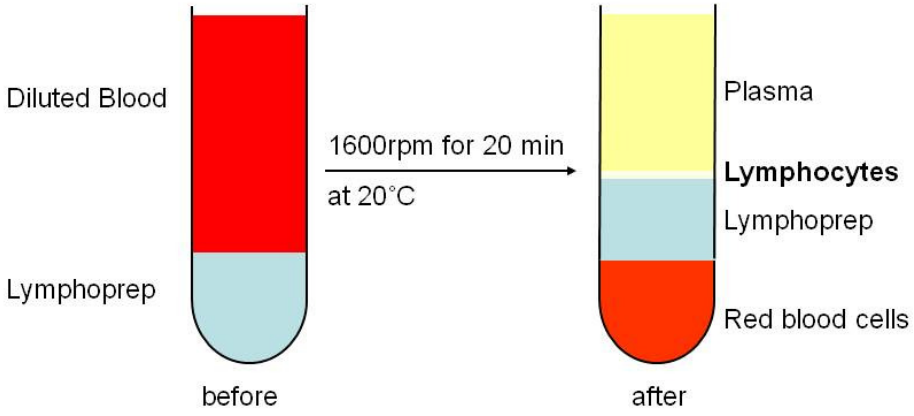


Figure 2.1: Use of Lymphoprep® to separate mononuclear cells from whole blood.

After centrifugation PBMCs are visible as thin layer in-between plasma and the Lymphoprep® solution.

2.4.2 **Freezing and thawing of cells**

Cell pellets from above were re-suspended in freezing medium (50% FCS, 40% RPMI, 10% Dimethyl Sulfoxide (DMSO)) and aliquots of 5-10x10^6/mL cells were frozen overnight at -80°C. After 24 hours cells were transferred to liquid nitrogen to store them until use.

Frozen PBMCs were gently thawed in a water bath at 37°C, washed twice with warm RPMI Medium (supplemented with 10% FCS; 1% penicillin/streptomycin (all from Gibco, UK) and 1% L-Glutamine (Lonza, UK) and centrifuged at 1500rpm for 5 minutes. Cells were then ready for further experiments.

2.4.3 **Generation of monocyte-derived macrophages**

Freshly isolated or thawed PBMCs were re-suspended at a concentration of 10^6/mL in RPMI medium (prepared as described above) into 24-well plates (Nunc, UK). Monocytes were allowed to adhere to the bottom of the plate for 1 hour at 37°C, non-adherent cells were then removed by vigorous washing with PBS. Monocyte-derived macrophages were generated as previously described (Buettner et al., 2005), kept in Opti-Mem I serum free medium (Life technologies, UK), and differentiated by adding 50ng/mL M-CSF (Peprotech, UK) per day. After 5 days of culture, the monocytes/macrophages were used for further experiments.

2.4.4 **Stimulation of cells**

Freshly isolated or thawed PBMC were re-suspended at a concentration of 10^6/mL in RPMI medium (prepared as described above) in 24-well plates and exposed to either Staphylococcus enterotoxin B (SEB) 1µg/mL or to Phorbol 12-myristate 13-acetate (PMA) 50ng/mL and Ionomycin (all from Sigma, UK)

1µg/mL for 5 or 16 hours at 37°C and 5% CO_2. After one hour, brefeldin A (Sigma, UK) 5µg/mL was added. Both ways of stimulation assessed the physiological cytokine production of already committed T cells. These stimulations do not drive naïve T cells into the T cell differentiation, but merely make visible already committed T cells.

2.4.5 Cell staining and Fluorescence activated cell sorting (FACS)

To identify certain T cell subsets, PBMCs were stained using primary antibodies conjugated with the fluorochromes PE, PE-Cy7, PerCP, FITC and Alexa Fluor 647 and analysed by flow cytometry.

For surface stainings ~10^6 cells were washed and re-suspended in 50µl of FACS buffer (PBS with 1% BSA) with fluorochrome-conjugated monoclonal antibodies specific for the respective cell surface protein and incubated for 30 minutes at 4°C. The antibodies used to stain surface molecules included anti-CD3PerCP, anti-CD3PE, anti-CD4PerCP, anti-CD4PE and anti-CD45ROPE-Cy7 (all from BD Bioscience, UK). To carry out additional intracellular stainings cells were fixed and permeabilized by using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, UK). Intracellular stainings were accomplished by using anti-IL-17Alexa Fluor 647- (eBioscience, UK), anti-IL21PE-, anti-IL22PE-, anti-IFN-γFITC- and anti RORγtPE-antibodies (all from BD Bioscience, UK).

After carrying out the staining procedures cells were washed and re-suspended in 500µl buffer for analysis.

Flow cytometry analysis was performed using an LSR II flow cytometer; data were analysed using FACS Diva software (BD Biosciences, UK) and FlowJo 8.7.3 (Treestar, US).

2.4.6 FlowCytomix™ Multiple Analyte Detection System

The FlowCytomix™ Multiple Analyte Detection System (eBioscience, UK) is a kit designed to quantify up to 20 target proteins simultaneously. The FlowCytomix™ technology uses fluorescent beads coated with specific antibodies to quantify multiple proteins in a single tube. The combination of two different bead sizes (4 and 5µm) and different internal fluorescent dye intensities makes it possible to distinguish up to 20 bead sets in one fluorescent channel. Streptavidin-PE, which binds to the biotin conjugate, emits at 578nm allowing the quantification of the analyte.

FlowCytomix™ bead-based assays follow the same principle as a sandwich immunoassay: In a first step antibody-coated beads are added to the sample to analyze. Then biotin-conjugated, antibodies specific for another epitope of the bound analytes are added. Streptavidin-PE is added for detection of biotin-conjugated antibody. The bead populations are finally analysed according to bead size and fluorescent signature by flow cytometry (Figure 2.2). Flow cytometry analysis was performed using an LSR II flow cytometer (BD Biosciences, UK). FlowCytomix™ Pro Software was used to calculate analyte concentrations in the samples.

For our experiments the Human Th1/Th2 11plex Ready-to-Use FlowCytomix Multiplex kit was used. This kit is designed for the measurement of human IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF-α and TNF-β. Human IL-17A/F and human IL-22 FlowCytomix™ Simplex for the simultaneous measurement of IL-17 and IL-22 were added.

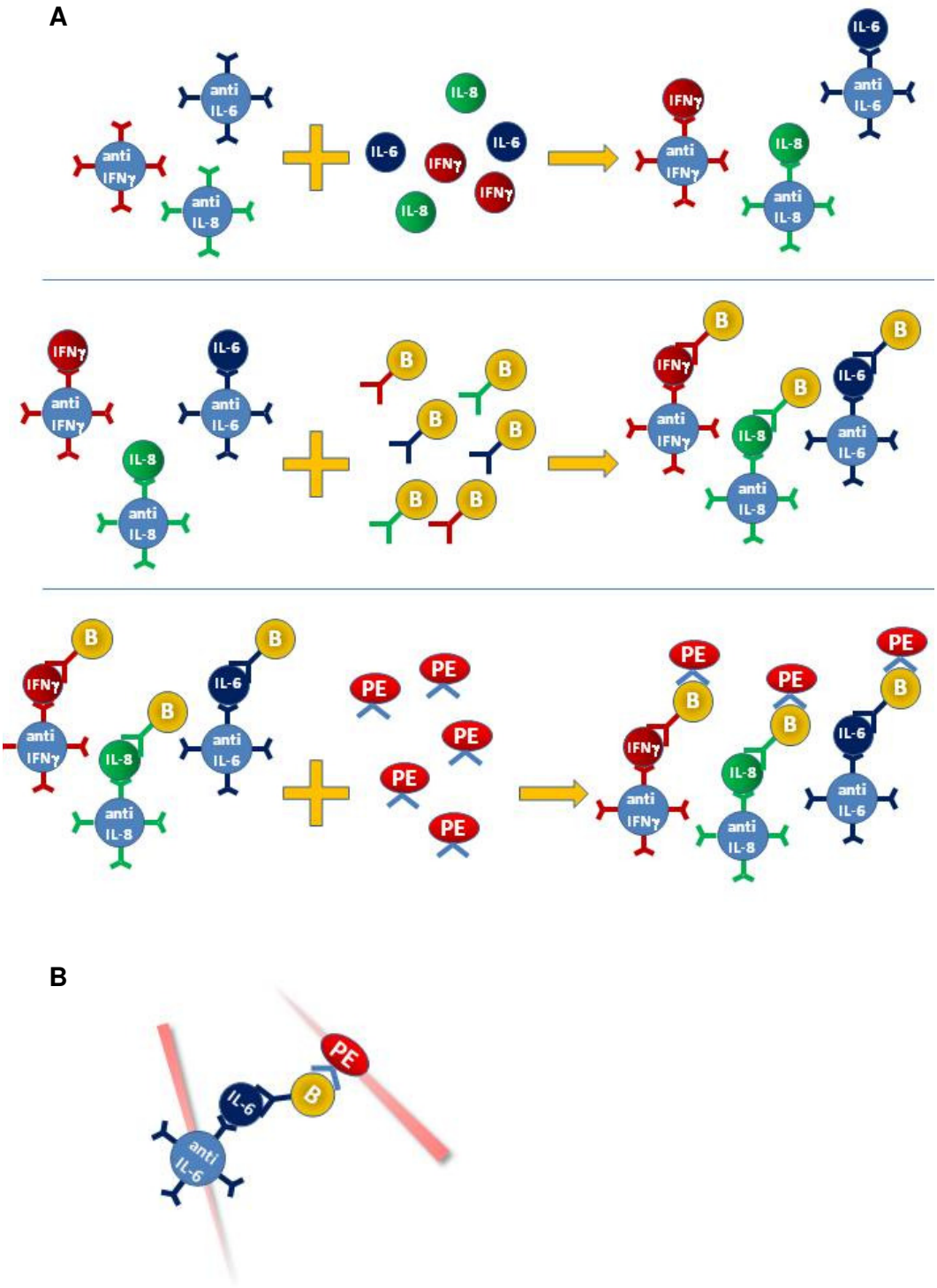


Figure 2.2: FlowCytomix™ bead-based assays

The bead based FlowCytomix™ Multiplex Immunoassays assays enables the parallel analysis of multiple analytes in short time. (A) Staining of cytokines (B) Flow cytometry is used to differentiate bead populations according to bead size and fluorescent signature. Figure modified after eBioscience FlowCytomix™ Multiple Analyte Detection User Guide.

Chapter 3 : Sequencing of *STAT3*

3.1 Introduction

In 2007, mutations in the signal transducer and activator of transcription 3 (*STAT3*) were shown to cause autosomal dominant Hyper IgE Syndrome (AD-HIES). Minegishi et al. described five different heterozygous *STAT3* mutations in eight out of fifteen unrelated non-familial AD-HIES patients. All mutations affected the DNA-binding domain of *STAT3* (Minegishi et al., 2007).

In parallel, Holland et al. sequenced *STAT3* in DNA samples from 50 AD-HIES patients and 48 unaffected relatives from 35 unrelated families. All 50 affected individuals carried heterozygous mutations in *STAT3* (Holland et al., 2007). The *STAT3* mutations were localized either to regions encoding the DNA-binding domain or the SH2 domain. It became apparent that most mutations cluster together and form mutational “hot spots”. All variations found were heterozygous missense mutations or in-frame deletions or duplications, associated with normal protein expression. The observed heterozygous mutations were shown to exert dominant-negative effects (Minegishi et al., 2007).

It has previously been shown that homozygous loss of *Stat3* leads to the death of deficient mouse embryos, whereas mice heterozygous for the *Stat3* mutations are phenotypically normal and fertile. Therefore, *Stat3* is necessary for survival, but haploinsufficiency does not result in an apparent phenotype in mice (Takeda et al., 1997). This finding is consistent with the observation that dominant-negative *STAT3* mutations decrease STAT3 homodimer activity to approximately 25% of the normal level (Minegishi and Karasuyama, 2009; Minegishi et al., 2007) (Figure 3.1).

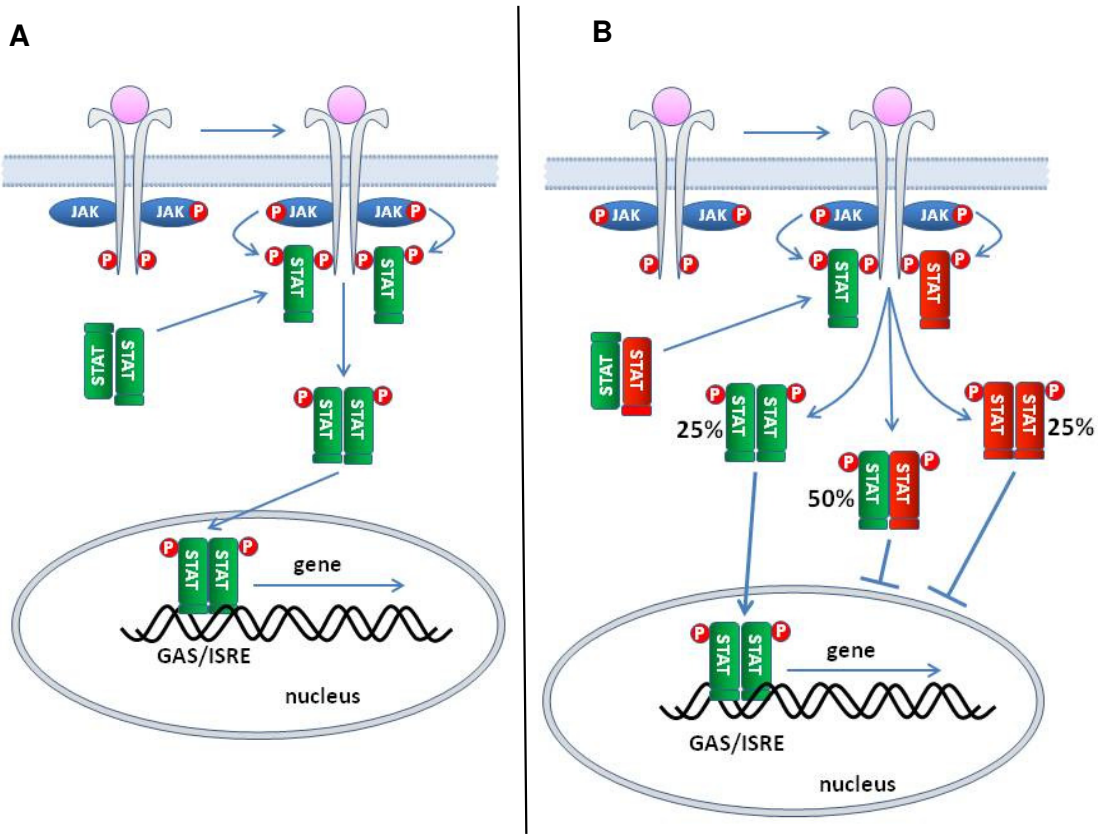


Figure 3.1: The JAK-STAT signalling pathway with heterozygous *STAT3* mutations

The observed heterozygous mutations have been shown to exert dominant-negative effects (Minegishi et al., 2007). Statistically, 75% of the STAT3 dimers are either heterozygous wild-type/mutated or homozygous mutated and thus not functional. The residual STAT3 activity (25%) might rescue the patients from early embryonic death, which is observed in Stat3-deficient mice (Takeda et al., 1997). (A) Pathway in normal condition. (B) Pathway with heterozygous mutated *STAT3*.

Since 2007, more than 200 patients carrying *STAT3* mutations, including 64 from this study, have been reported in literature either in individual case reports or national and international studies (Anolik et al., 2009; Avery et al., 2008, 2010; de Beaucoudrey et al., 2008; Chandesris et al., 2012; Holland et al., 2007; Jiao et al., 2008; Kim et al., 2009; Kumánovics et al., 2010; Liu et al., 2011b; Ma et al., 2008; Milner et al., 2008; Minegishi et al., 2007; Powers et al., 2009; Renner et al., 2007, 2008; Schimke et al., 2010; Vinh et al., 2010; Woellner et al., 2010; Xie et al., 2010) (Figure 3.2). Nevertheless not all patients with the clinical diagnosis of AD-HIES present mutations in *STAT3* (Minegishi et al., 2007; Renner et al., 2008; Woellner et al., 2010).

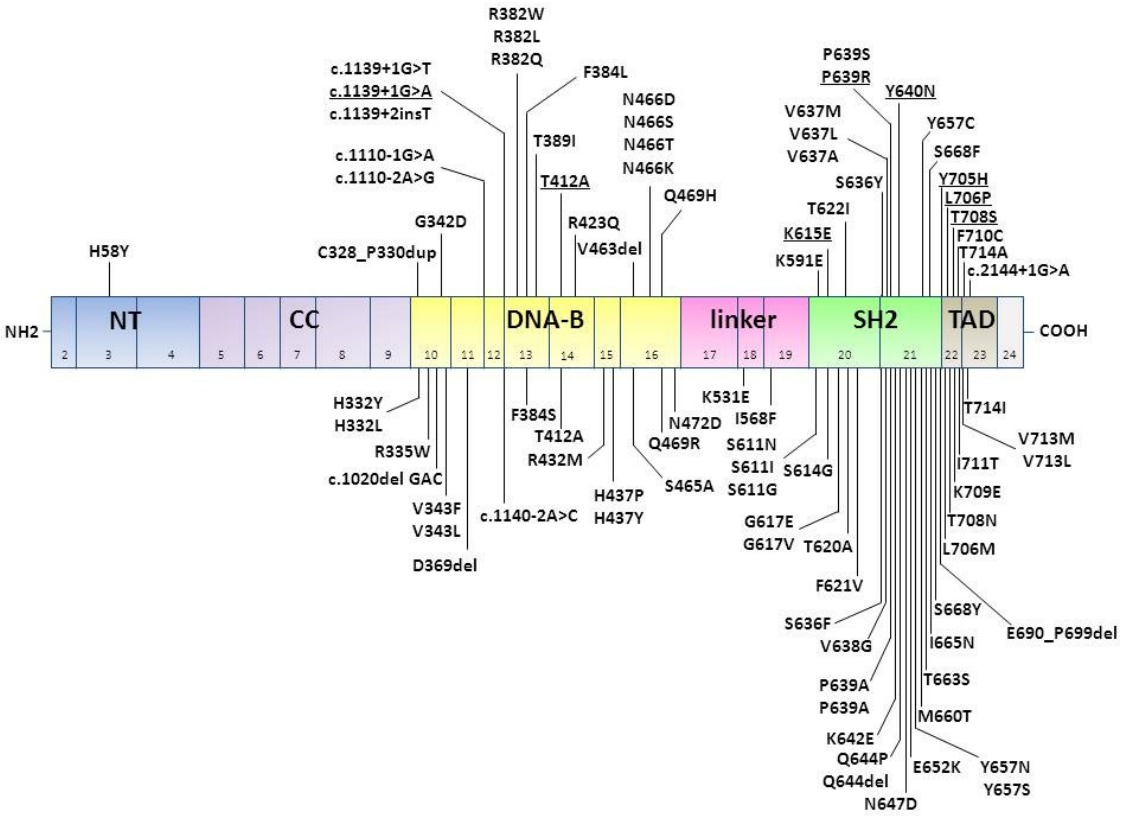


Figure 3.2: Schematic structure of STAT3 revealing mutational hotspots in the DNA-binding and SH2 domain

Schematic representation of STAT3 showing the positions of the *STAT3* mutations. Mutations affect the amino-terminal (NT), the DNA-binding (DNA-B), the linker, the Src homology 2 (SH2) and the transcriptional activation (TAD) domains. Mutations on the upper part of the cartoon were identified in patients of the current study (Woellner et al., 2010). Underscored mutations have not yet been published in literature. Figure modified after Chandesris et al., 2012.

3.2 Questions

1) Do all Patients with the clinical diagnosis of AD-HIES carry mutations in *STAT3*? And how common is a diagnosis of AD-HIES without a *STAT3* mutation?
2) Do all mutations affect the DNA-binding domain or the SH2-domain of *STAT3* or are there other domains hit by mutations?
3) Is there segregation of the mutations with the affection status of family members in AD-HIES families with more than one affcted patient?
4) What is the bioinformatic assessment of STAT3 missense changes using the online prediction tool PolyPhen?

3.3 Results

3.3.1 *STAT3* mutations

We amplified and sequenced all coding parts of *STAT3* in 153 patients with suspected AD-HIES. All patients showed normal PCR amplification for all coding exons (exons 2-24; exon 1 is not coding) analysed, and all PCR products had the expected length. PCR products of a representative patient are shown in Figure 3.3.

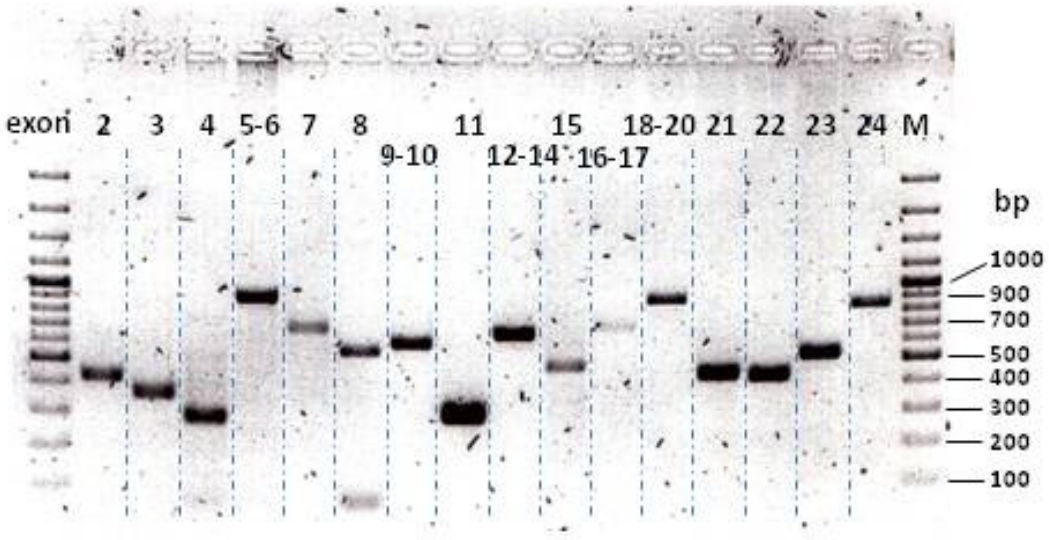


Figure 3.3: *STAT3* PCR products

Agarose gel of amplified *STAT3* PCR products for one patient with suspected AD-HIES. Exons 9 and 10 as well as exons 12 to 14 and exon 16 and 17 are located in close proximity on the genomic DNA and were amplified in one PCR product. All amplified products showed the expected length. Although the band of exon 16 and 17 is fainter than the others it was good enough for sequencing. M, marker; bp, base pairs.

Out of the 153 patients sequenced, 82 carried heterozygous mutations in *STAT3*. Seventy-one patients did not show any mutation in the coding regions of *STAT3* or their flanking intronic sequences. Overall, we found 39 different mutations: Fifty-five patients carried mutations described previously, (de Beaucoudrey et al., 2008; Holland et al., 2007; Jiao et al., 2008; Ma et al., 2008; Milner et al., 2008; Minegishi et al., 2007; Renner et al., 2008) 27 patients harboured yet undescribed, novel mutations.

The mutations affected the DNA-binding domain, the SH2 domain, the transcriptional activation domain, and the N-terminal domain. Seventy-two mutations were missense mutations in which a single nucleotide was replaced, resulting in an amino acid exchange, one patient harboured an in-frame duplication of three amino acids and one patient carried an in-frame deletion of one amino acid.

Six mutations, seen in eight patients, were heterozygous mutations at the intron-exon boundaries of exons 12 or 22, affecting the DNA-binding and transcriptional activation domain, respectively. Each of these intronic mutations led to an incorrect splicing of its adjacent exon (Table 3.1).

None of the mutations identified in our patients was observed in 100 healthy controls or reported in any of the SNP databases.

A substantial portion (71 patients) of our cohort did not have mutations in the coding or splice regions of *STAT3*. To exclude undetected splice site variants not yet described in healthy individuals, we sequenced the cDNA from 25 of these patients, none of whom revealed any mutation or exon skipping. Because of the limited availability of samples, the cDNA of the other patients was not examined.

Number of patients	Protein domain	Site of mutation	DNA sequence change	Predicted amino acid change
1	N-terminal	Exon 3	c.172C>T	H58Y
1	DNA-binding	Exon 10	c.982_990dupTGCATGCCC	C328_P330dup
1	DNA-binding	Exon 10	c.1025G>A	G342D
3	DNA-binding	Intron 11	c.1110–2A>G	D371_G380del
1	DNA-binding	Intron 11	c.1110–1G>A	D371_G380del
1	DNA-binding	Intron 12	c.1139+1G>T	D371_G380del
1	DNA-binding	Intron 12	c.1139+1G>A	D371_G380del
1	DNA-binding	Intron 12	c.1139+2insT	D371_G380del
16	DNA-binding	Exon 13	c.1144C>T	R382W
1	DNA-binding	Exon 13	c.1145G>T	R382L
11	DNA-binding	Exon 13	c.1145G>A	R382Q
1	DNA-binding	Exon 13	c.1150T>A	F384L
2	DNA-binding	Exon 13	c.1150T>C	
1	DNA-binding	Exon 13	c.1166C>T	T389I
1	DNA-binding	Exon 14	c.1234A>G	T412A
1	DNA-binding	Exon 14	c.1268G>A	R423Q
1	DNA-binding	Exon 16	c.1387_1389delGTG	V463del
1	DNA-binding	Exon 16	c. 1396 A>G	N466D
1	DNA-binding	Exon 16	c.1397A>G	N466S
1	DNA-binding	Exon 16	c.1397A>C	N466T
1	DNA-binding	Exon 16	c.1398C>G	N466K
1	DNA-binding	Exon 16	c.1407G>T	Q469H
1	SH2	Exon 20	c.1771A>G	K591E
2	SH2	Exon 20	c.1843A>G	K615E
1	SH2	Exon 20	c.1865C>T	T622I
1	SH2	Exon 21	c.1907C>A	S636Y
12	SH2	Exon 21	c.1909G>A	V637M
1	SH2	Exon 21	c.1909G>T	V637L
1	SH2	Exon 21	c.1910T>C	V637A
1	SH2	Exon 21	c.1915C>T	P639S
1	SH2	Exon 21	c.1916C>G	P639R
1	SH2	Exon 21	c.1918T>A	Y640N
2	SH2	Exon 21	c.1970A>G	Y657C
1	SH2	Exon 21	c.2003C>T	S668F
1	TAD	Exon 22	c.2113T>C	Y705H
1	TAD	Exon 22	c.2117T>C	L706P
2	TAD	Exon 22	c.2124C>G	T708S
1	TAD	Exon 22	c.2129T>C	F710C
1	TAD	Exon 22	c.2141C>G	T714A
1	TAD	Intron 22	c.2144+1G>A	p.?

Table 3.1: *STAT3* mutations of the 153 AD-HIES patients studied

Of the 153 patients studied, 82 patients had 39 distinct mutations. Fourteen of these mutations were reported previously (Minegishi et al, 2007; Holland et al, 2007; Renner et al, 2008; Milner et al, 2008; Ma et al, 2008; de Beaucoudrey et al 2008 and Jiao et al, 2008) The other 25 mutations, which are shaded in this table, were not reported at the time of discovery. TAD, transcriptional activation domain; p.?, unknown effect on protein level.

3.3.2 Mutation in the N-terminal domain

The N-terminal domain, comprising approximately 130 amino acids, is conserved among the STATs and represents an independently folded and stable moiety (Kisseleva et al., 2002). It has been shown that the N-terminal domain is needed for the formation of unphosphorylated STAT3 dimers and the nuclear accumulation of STAT3 upon phosphorylation (Vogt et al., 2011).

Only one patient of our cohort carried a heterozygous mutation in the N-terminal domain of STAT3. The mutation affects base pair 172 (C>T) and leads to the amino acid exchange histidine (H) to tyrosine (Y) at position 58 (H58Y) (Figure 3.4).

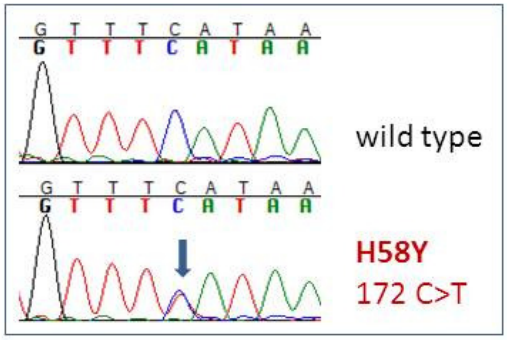


Figure 3.4: Chromatogram of the mutation affecting the N-terminal domain of STAT3

The chromatogram represents a wild-type sequence and the heterozygous change of the cytosine to thymine at position 172 of the patient indicated with a blue arrow. The mutation leads to the amino acid exchange H58Y.

3.3.3 Mutations in the DNA-binding domain

Most mutations were found in the DNA binding domain of STAT3. Forty-nine patients carried 20 different mutations. The majority of mutations affected the arginine (R) residue at position 382 in exon 13. In 16 cases, the arginine was replaced by tryptophan (W), in 11 patients by glutamine (Q) and in one patient with leucine (L). Beside the arginine 382 two further amino acid residues were shown to be mutated in exon 13. The phenylalanine (F) at position 384 was changed to leucine (L) in three patients and threonine (T) at position 389 was replaced by isoleucine (I) in one case.

We also detected mutations in exons 10, 14, and 16.

One patient harboured an in-frame duplication of three amino acids (C328_P330dup) in exon 10 as shown in Figure 3.5, another patient was found to carry the replacement from glycine to aspartic acid at position 342 (G342D).

Two patients had mutations affecting exon 14. One carried the change from threonine (T) to alanine (A) at position 412, another the replacement from arginine (R) 423 to glutamine (Q).

Four of the six mutations detected in exon 16 resulted in a replacement of asparagine (N) residue at position 466; four patients carried four different changes, one to aspartic acid (D), one to serine (S), one to threonine (T) and one to lysine (K). The deletion of the valine (V) residue at position 463 (V463del) and the change glutamine from (Q) to histidine (H) at position 469, were both found in one patient respectively (Figure 3.6 and 3.7). Five mutations of the DNA binding domain observed in eight patients are splice-site mutations and are discussed in chapter 3.3.6.

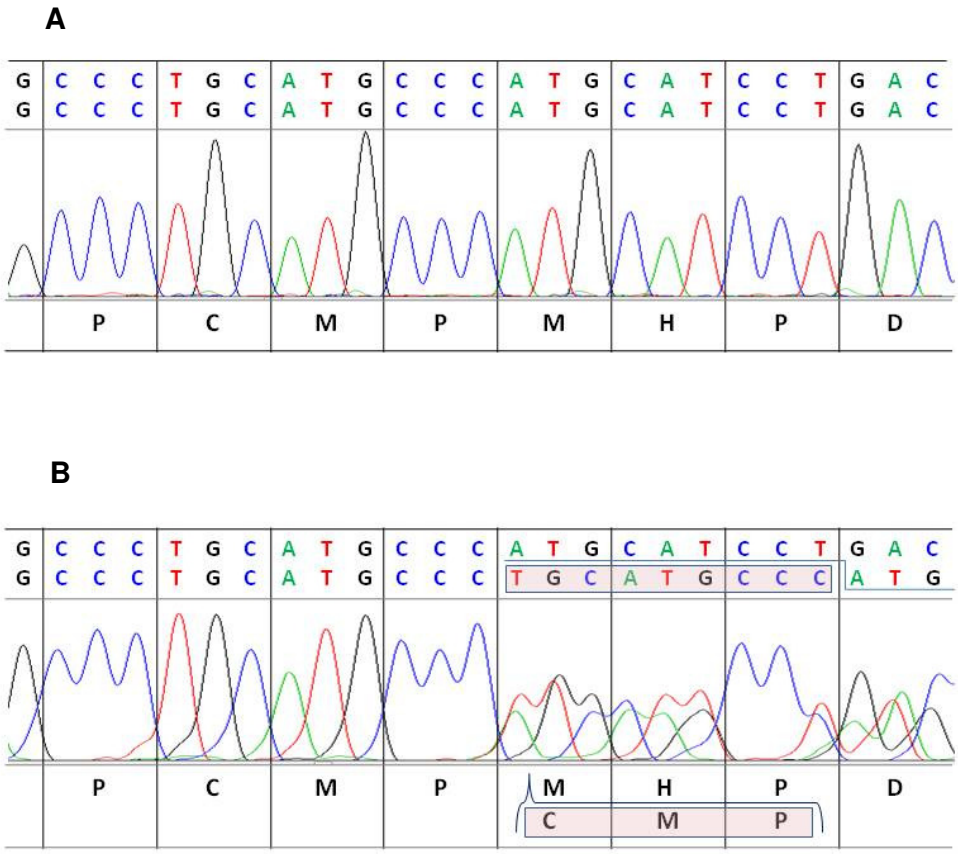


Figure 3.5: Chromatogram of duplication mutation in exon 10

Chromatogram of exon 10 of *STAT3* showing the nucleotides, the respective peaks and the resulting protein sequence. (A) Wild-type sequence (B) Sequence of AD-HIES patient with heterozygous duplication and insertion of 9 nucleotides (c.982_990dupTGCATGCCC) resulting in an overlap of chromatograms. The duplicated nucleotide and the resulting duplicated amino acid sequences are shaded.

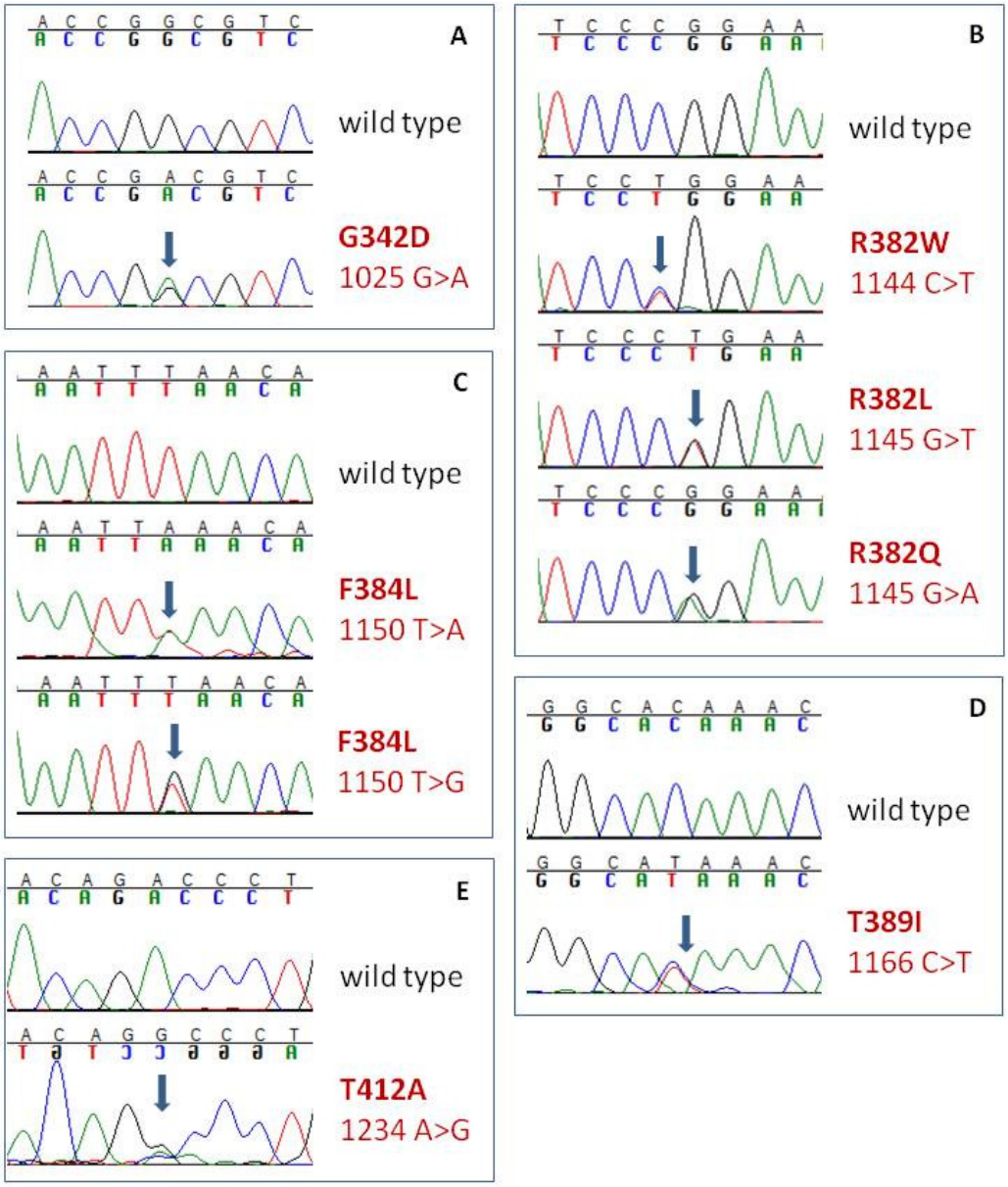


Figure 3.6: Chromatograms of the STAT3 DNA-binding domain mutations -part I

The chromatograms represent wild-type sequences and the heterozygous changes (A) 1025G>A leading to amino acid substitution G342D (B) 1144C>T, 1145G>T and 1145G>A leading to amino acid changes R382W, R382L and R382Q (C) 1150T>A and 1150T>G both leading to the changes F384L (D) 1166C>T leading to the substitution T389I and (E) 1234A>G leading to the change T412A. All changes are highlighted by a blue arrow.

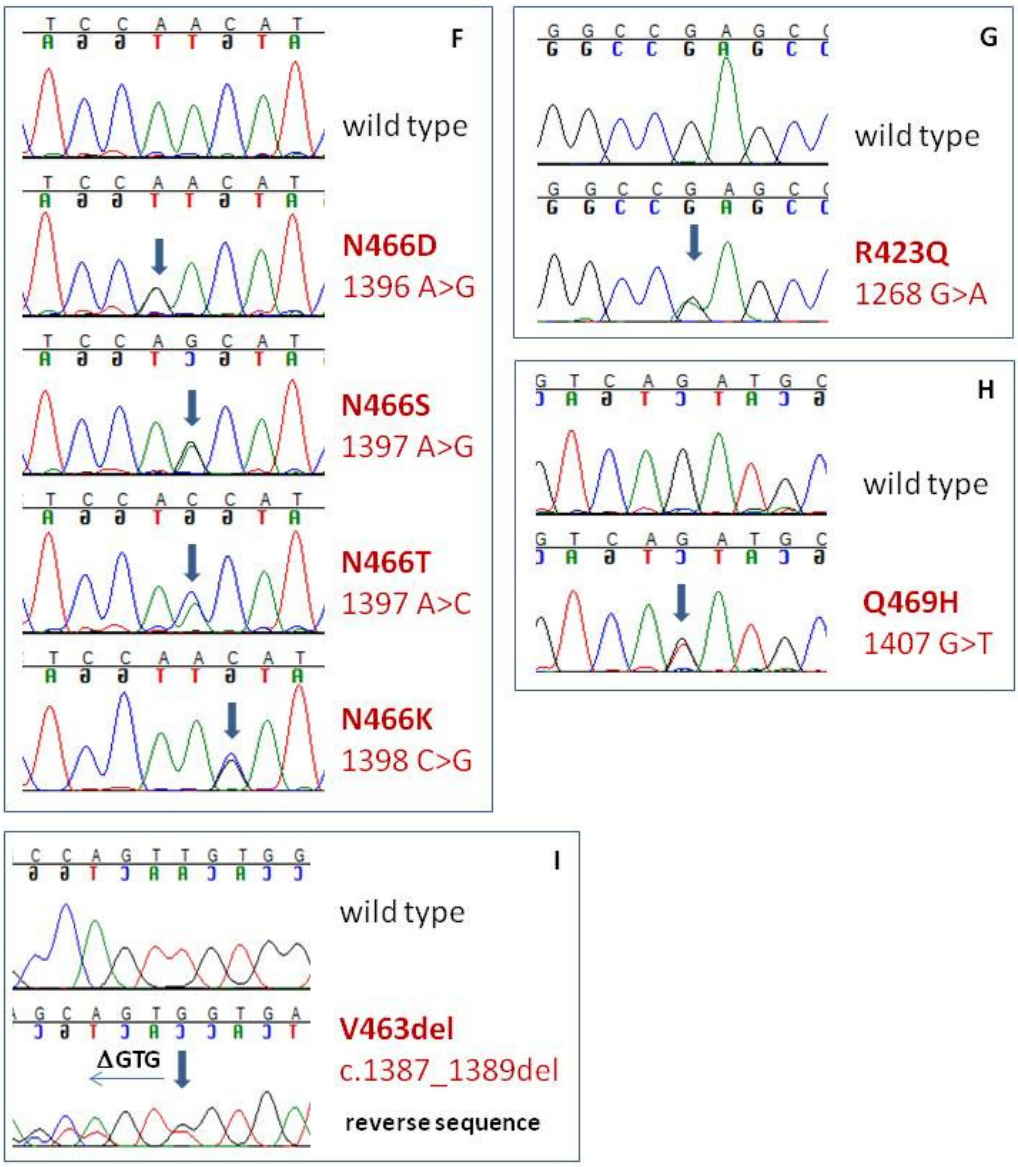


Figure 3.7: Chromatograms of the STAT3 DNA-binding domain mutations -part II

The chromatograms represent wild-type sequences and the heterozygous changes (F) 1396A>G, 1397A>G, 1397A>C and 1398C>G leading to the amino acid changes N466D, N466S, N466T and N466K (G) 1268G>A leading to the substitution R423Q (H) 1407G>T leading to the change Q469H and (I) c.1387_1389del resulting in the deletion of the Valine (V) at position 463 (V463del) (the heterozygous deletion of the three nucleotides results in an overlap of the chromatograms). The chromatogram shows a reverse sequence. All changes are indicated by a blue arrow.

3.3.4 **Mutations affecting the Src homology 2 (SH2) domain**

Twenty-five patients had twelve different mutations in the SH2 domain: the most frequent one, with valine (V) replaced by methionine (M) at position 637, was identified in twelve patients. In two other patients, the valine was replaced by leucine (L) and alanine (A) respectively. Several other amino acids in exon 21 were also shown to be mutated. The serine (S) at position 636 and 668 were replaced by tyrosine (Y) and phenylalanine (F); and tyrosines (T) 640 and 657 were replaced by asparagine (N) and cystein (C) respectively. The proline (P) at position 639 showed two different changes one to serine (S) and one to arginine (R).

Three other mutations affecting the SH2 domain were found in exon 20. At positions 591 and 615 the lysines (K) were found to be substituted by glutamic acid (E) and at position 622, threonine (T) was replaced by Isoleucine (I) (Figure 3.8 and 3.9).

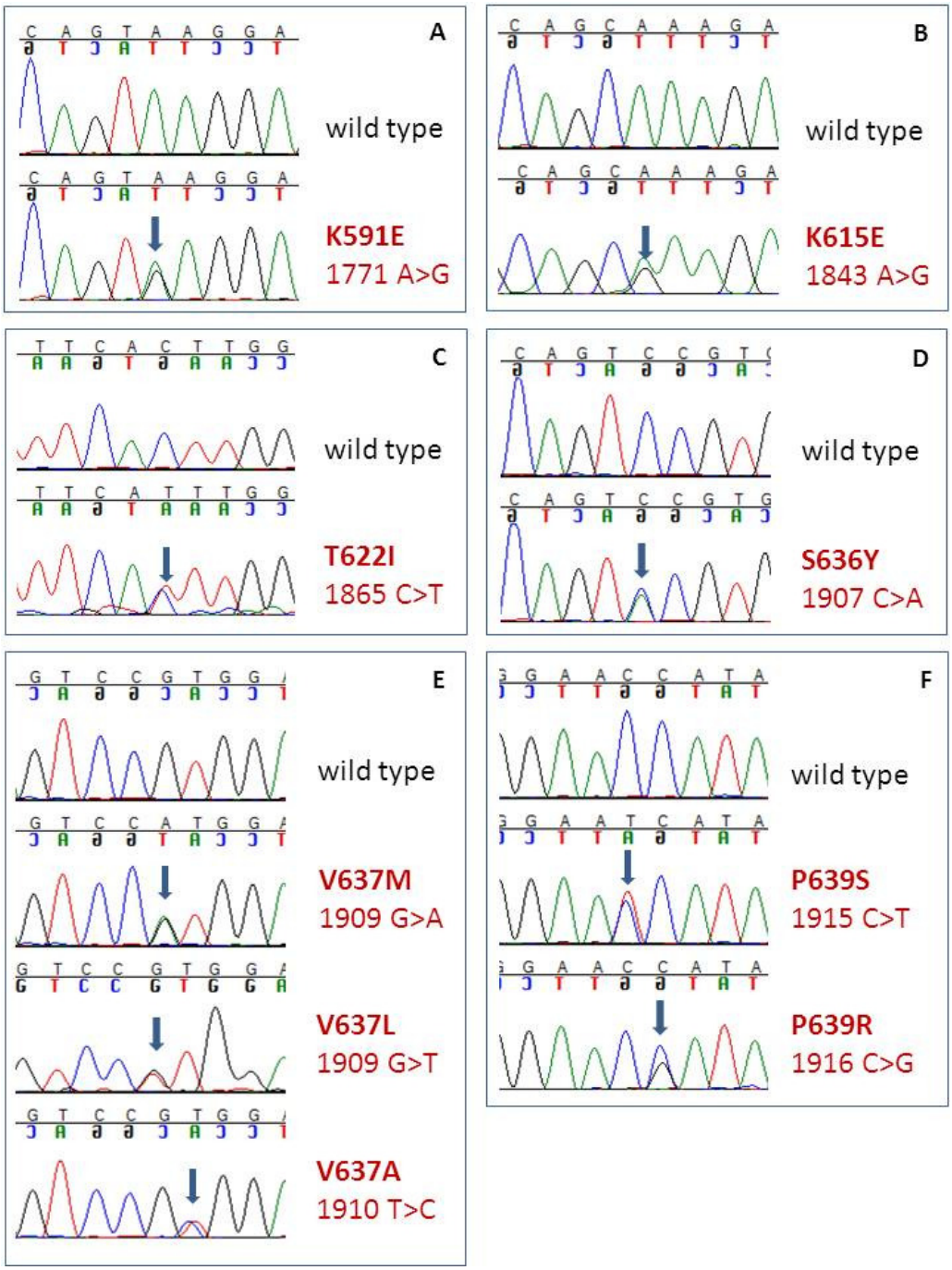


Figure 3.8: Chromatograms of the STAT3 SH2-domain mutations -part I

The chromatograms represent wild-type sequences and the heterozygous changes (A) 1771A>G leading to amino acid substitution K591E (B) 1843A>G leading to the change K615E (C) 1865C>T ending in the substitution T6221I (D) 1907C>A leading to amino acid substitution S636Y (E) 1909G>A, 1909G>T and 1910T>C leading to amino acid changes V637M, V637L and V637A and (F) 1915C>T and 1916C>G leading to the substitutions P639S and P639R respectively. All changes are highlighted by a blue arrow.

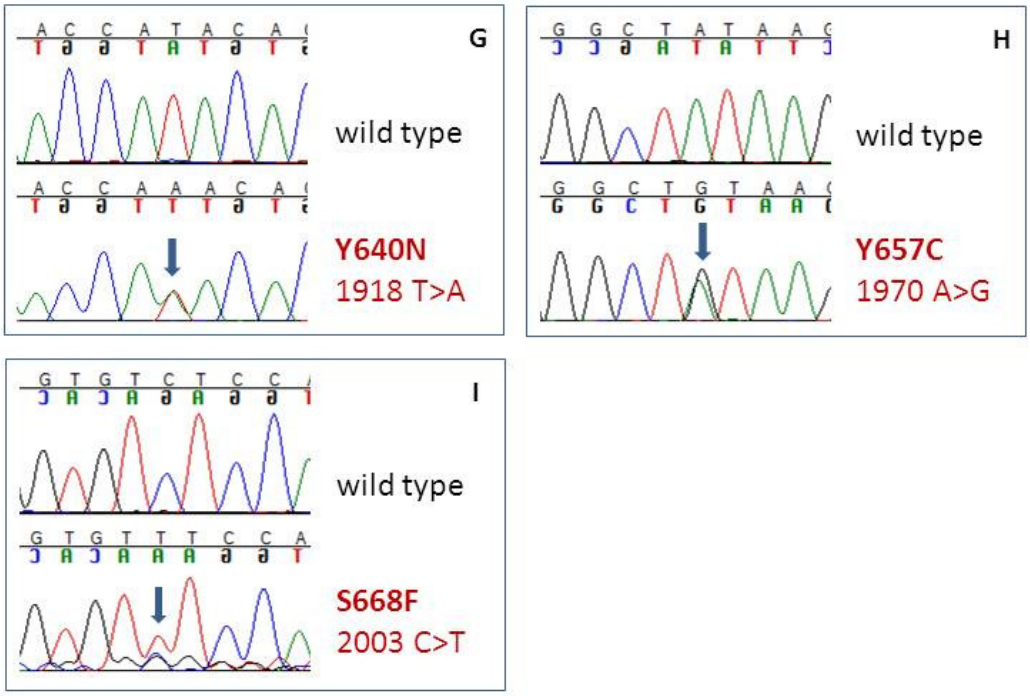


Figure 3.9: Chromatograms of the STAT3 SH2-domain mutations -part II

The chromatograms represent wild-type sequences and the heterozygous changes (G) 1918T>A leading to amino acid substitution Y640N (H) 1970 A>G leading to the change Y657C and (I) 2003C>T ending in the substitution S668F. All changes are highlighted by a blue arrow.

3.3.5 Mutations affecting the transcriptional activation domain

Seven patients had six different mutations in the transcriptional activation domain, which is downstream of the SH2 domain. Five of these were missense mutations whereas one affected the splice site of exon 22.

All mutations in this domain were located in exon 22. The amino acids found to be changed were the tyrosine (Y) at position 705 substituted by histidine (H), the leucine (L) at position 706 changes to proline (P), the threonines (T) 708 and 714 replaced by serine (S) and alanine (A) respectively and the phenylalanine (F) at position 710 substituted by cysteine (C) (Figure 3.10).

One mutation affecting the transcriptional activation domain is a splice-site mutation and discussed in chapter 3.3.6.

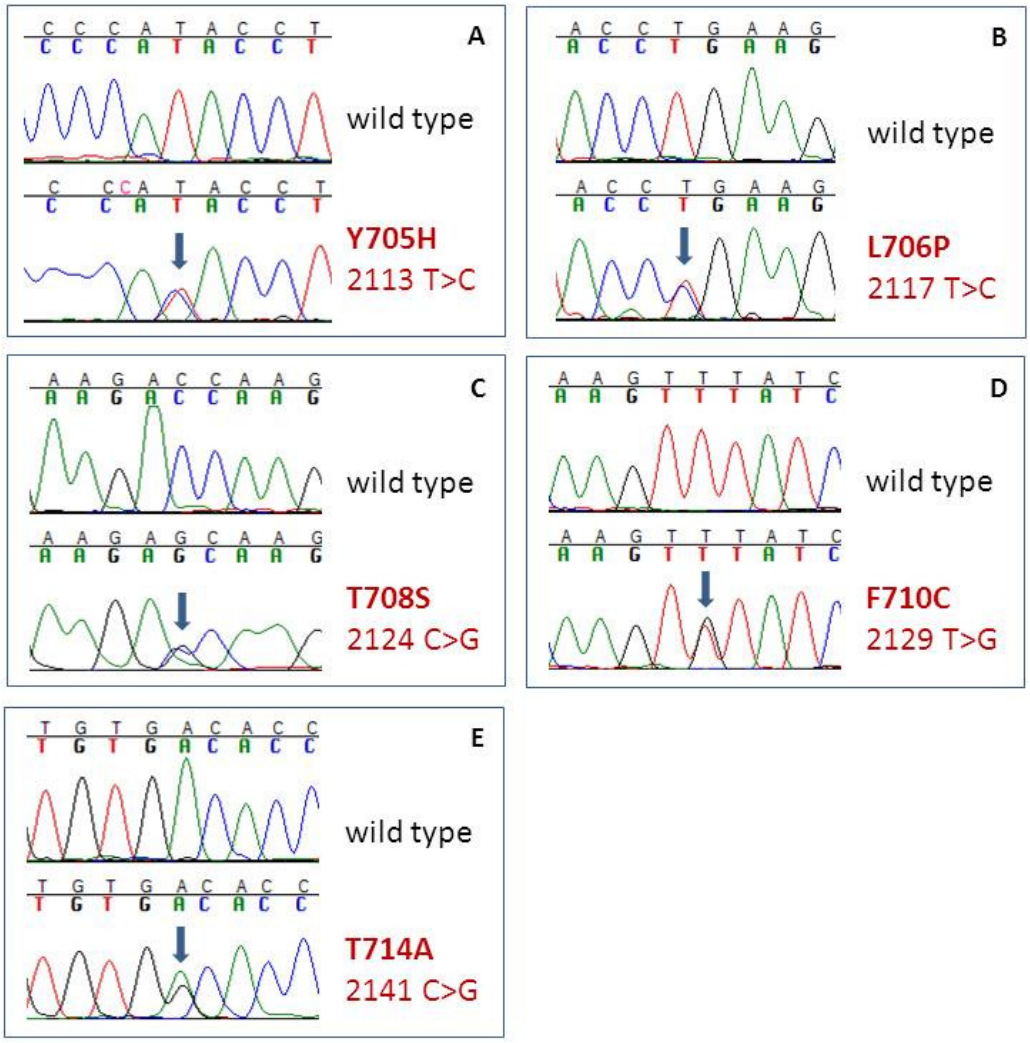


Figure 3.10: Chromatograms of the STAT3 mutations affecting the transcriptional activation domain

The chromatograms represent wild-type sequences and the heterozygous changes (A) 2113T>C leading to amino acid substitution Y705H (B) 2117T>C leading to the change L706P (C) 2124C>G ending in the substitution T708S (D) 2129T>G leading to amino acid substitution F710C and (E) 2141G>A leading to amino acid change T714A. All changes are highlighted by a blue arrow.

3.3.6 Splice site mutations

Although more than 90% of the mutations were located in the exons of *STAT3*, we found 6 mutations at the intron-exon boundaries.

Four patients had 2 different heterozygous mutations at the 3′ splice site before exon 12, and 3 patients had distinct mutations in at the 5′ splice site after exon 12. All 5 mutations caused the skipping of exon 12 leading to a heterozygous in-frame deletion of amino acids 371 to 380 (Figure 3.11). The deletion of the exon has been confirmed by sequencing the cDNA as shown in Figure 3.12.

One patient harboured a mutation one nucleotide after exon 22 that caused the exon to be skipped. The effect of this splice site mutation is complex. In healthy individuals, alternative splicing of exon 23 results in 2 major isoforms of STAT3: STAT3α and STAT3β. Whereas STAT3α contains the entire exon 23, STAT3β lacks the first 50 bp of the same exon (Maritano et al., 2004). The splice site introduced by the mutation causes the 43-bp exon 20 to be skipped. In the wild-type STAT3α transcript, this deletion would result in a frame shift and possibly nonsense mediated decay. However, in conjunction with the shortened exon 23 of STAT3β, this deletion is predicted to produce a transcript coding for STAT3α with an in-frame deletion of amino acids 701 to 732, which would encompass both the tyrosine and serine phosphorylation sites of STAT3α (Figure 3.13).

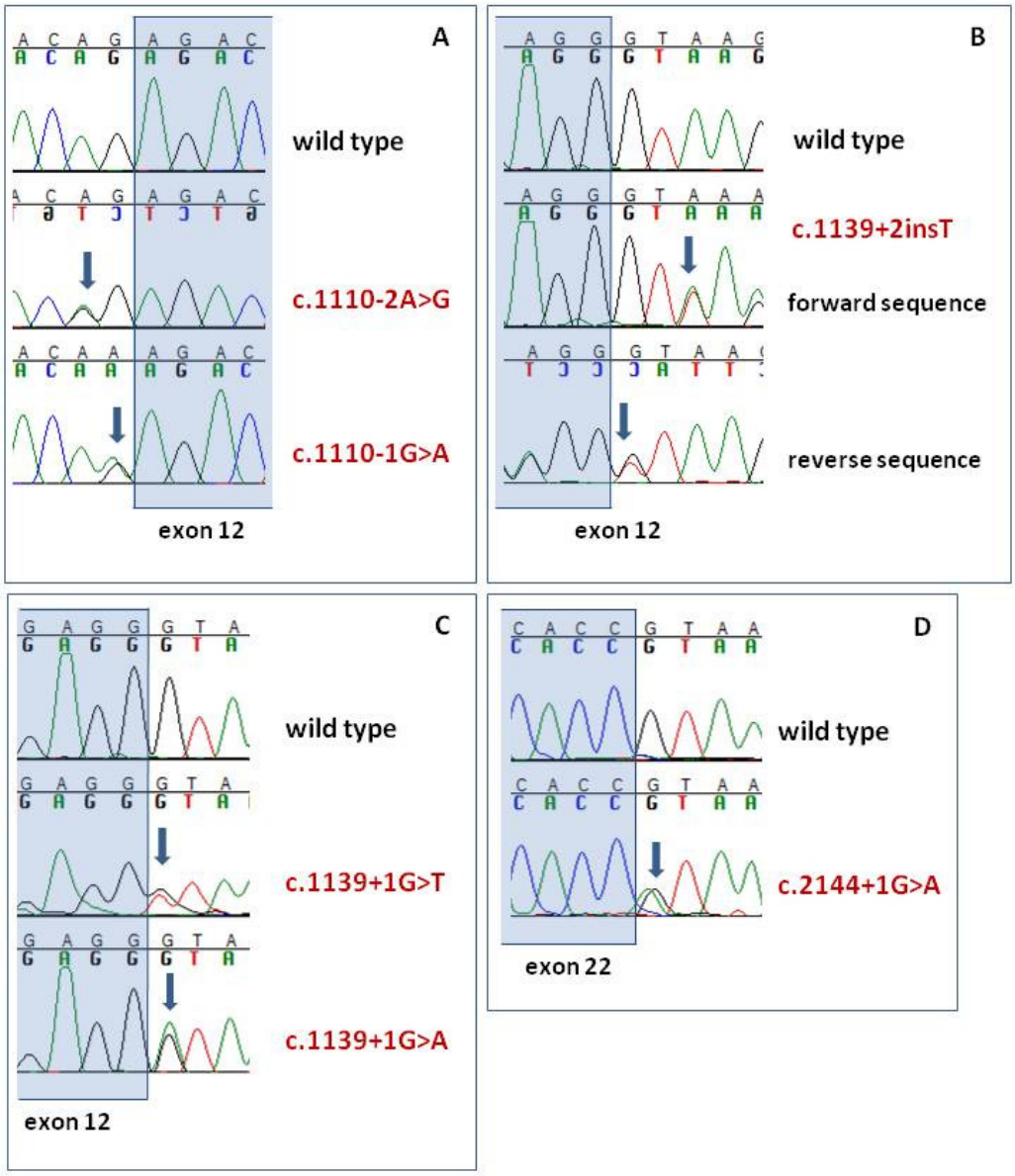


Figure 3.11: Splice site mutations on gDNA

The chromatograms represent wild-type sequences and the heterozygous splicesite mutations (A) c.1110-2A>G and c.1110-1G>A in intron 11 (B) c.1139+2insT sequenced in forward and reverse direction in intron 12 (C) 1139+1G>T and 1139+1G>A in intron 12 and (D) 2144+1G>A in intron 23. The contiguous exons are highlited. All changes are highlighted by a blue arrow.

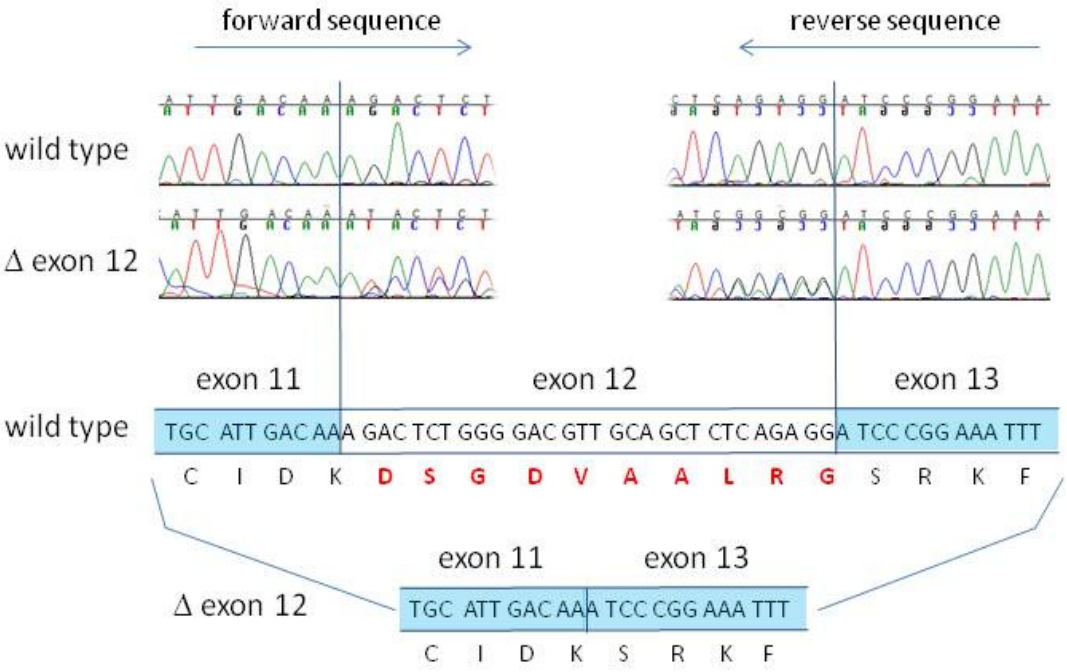


Figure 3.12: Skipping of exon 12

cDNA sequence and schematic representation of the consequence of the mutations detected in the splice site regions of exon 12. The cDNA sequences show wild-type sequences of healthy controls and the overlap of chromatograms in patients lacking exon 12 on one allele. The cDNA has been sequenced in both directions (forward and reverse). The identified splicing mutations cause an in-frame deletion of 10 amino acids (red).

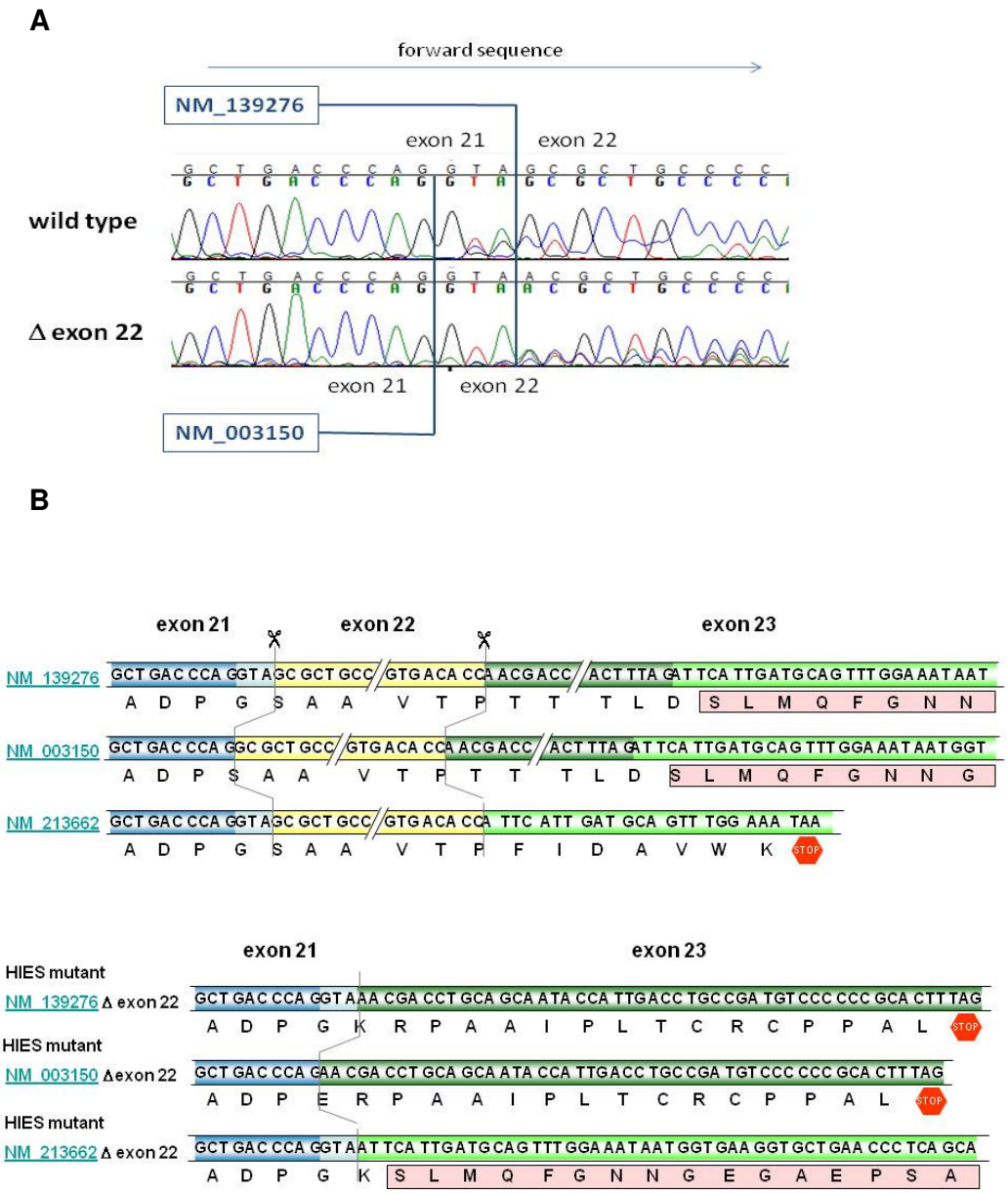


Figure 3.13: Splice site mutation exon 22

Two alternative splicing sites, one between exons 21 and 22 and one between exons 22 and 23, result in three alternative wild-type transcripts: two STAT3α transcripts (NM_139276, NM_003150) and one STAT3β (NM_213662) transcript. The splice site introduced by the mutation 1 nucleotide after exon 22 causes the 43-bp exon 22 to be skipped. In mutant variants of NM_139276 and NM_003150, this deletion would result in a frame shift and a premature stop-codon. In conjunction with the 50-bp shorter exon 23 of STAT3β, the deletion of exon 22 is predicted to produce a transcript that codes for a variant of STAT3α with an in-frame deletion of 31 amino acids. (A) Chromatogram of healthy control and patient. (B) Schematic representation of the consequences of the splicing mutation. The amino acid sequences of the STAT3α transcripts shared with the mutated STAT3β transcript are highlighted in *pink*.

3.3.7 **Familial cases**

Nine of the 153 AD-HIES patients referred to us had affected family members. Sequencing of these family members showed that they carried the same mutation as the probands. The pedigrees of the nine families are shown in Figure 3.14. In three cases (UPN60, UPN110 and UPN160) the mutations have been passed on to more than one offspring. In all three families, there is perfect segregation of the mutations with the affection status of the family members. The mutations inherited are the two point mutations F384L and G342D, and the splice site mutation c.1139+1G>A (Figure 3.15). In other five cases (UPN2, UPN19, UPN94, UPN106 and UPN153) there has been inheritance from the patient to just one offspring. These mutations include the point mutation H58Y affecting the N-terminal domain, R382W and N466S in the DNA-binding domain and V637M and Y640N in the SH2 domain. In one family (UPN65) two siblings carry the same mutation. It is likely that both inherited the mutation from the mother who presented similar symptoms but died before definitive diagnosis.

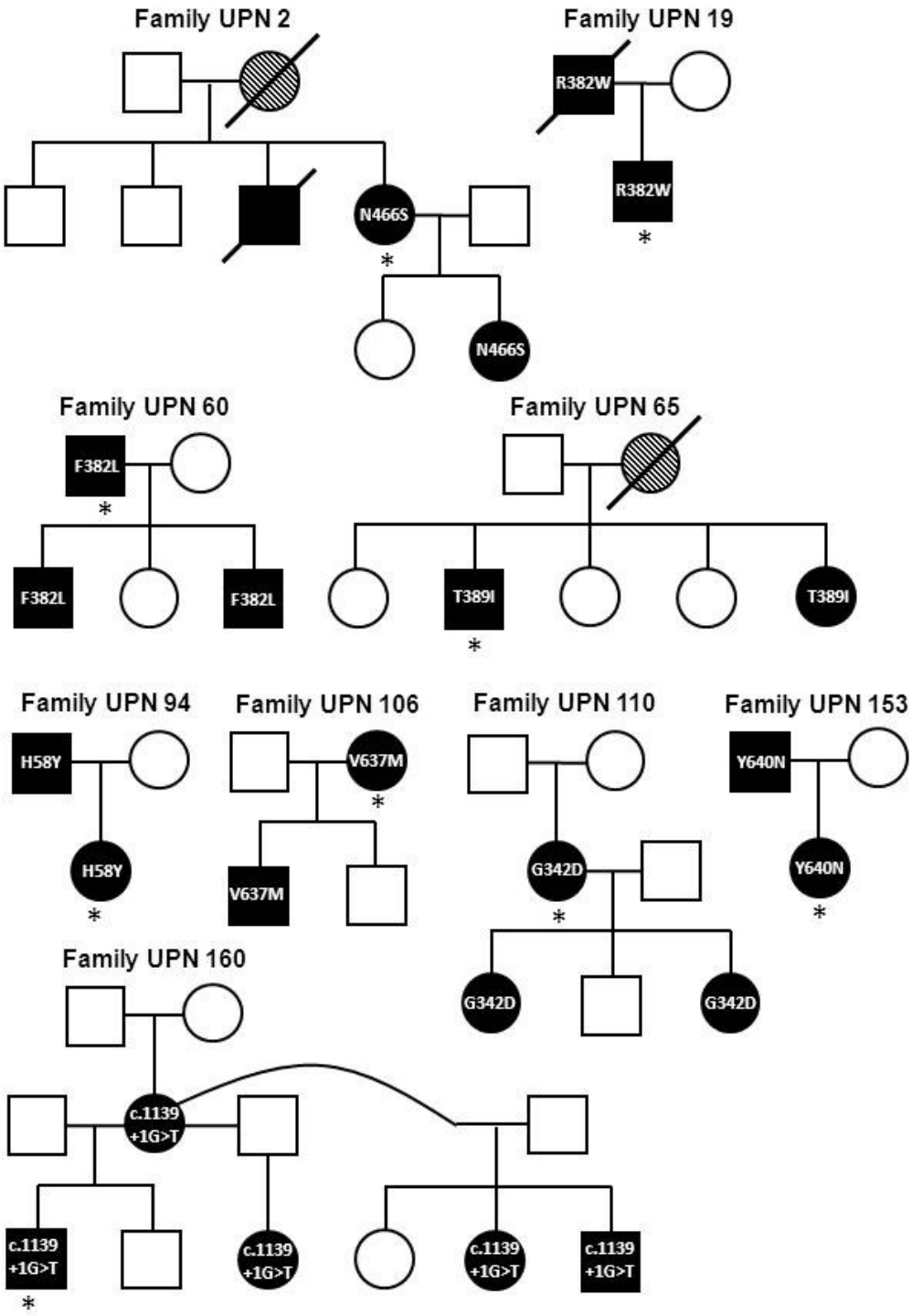


Figure 3.14: Pedigrees of familial AD-HIES cases

Nine AD-HIES patients had affected family members. All affected family members showed the same mutation as the probands. Squares indicate males; circles, females. Filled symbols, patients; slashes, deceased individuals. The shaded square indicate possibly affected persons. UPN, unique personal number. Probands are highlighted by asterisks.

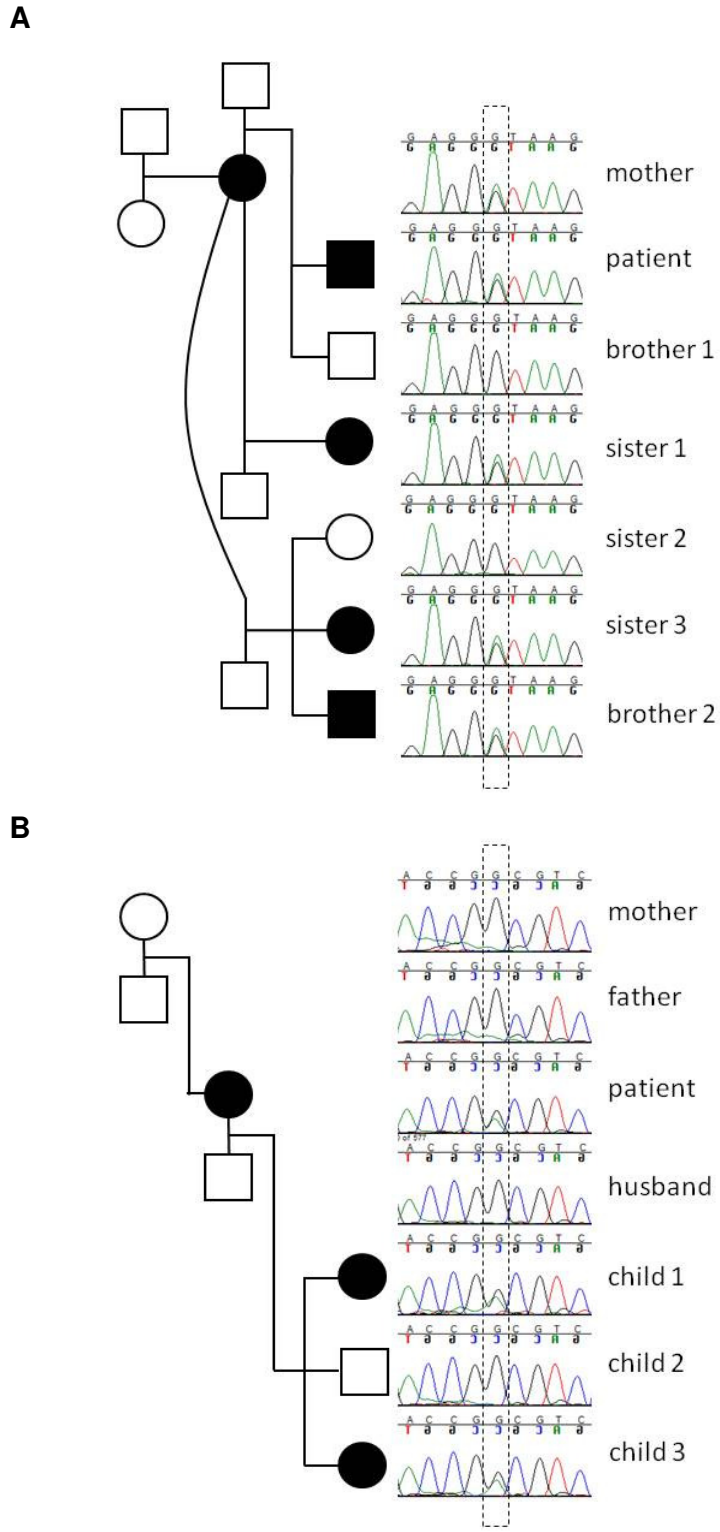


Figure 3.15: Pedigrees and chromatograms of two familial AD-HIES cases

Pedigrees and the relative chromatograms of two AD-HIES families in which the mutations segregate perfectly with the affection status. The family depicted in (A) carries the splice site mutation c.1139+1G>T leading to the heterozygous in-frame deletion of exon 12. The family shown in (B) carries the point mutation c.1025G>A leading to the amino acid change G324D in the DNA-binding domain of STAT3. Squares indicate males; circles, females. Filled symbols, patients.

3.3.8 Impact of amino acid substitutions

To assess the severity of the effect of the amino acid changes found on the structure and function of the STAT3 protein the online prediction tool PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) was used. All 31 different missense mutations found were analyzed using the Ensembl transcript STAT3-001 ENST00000264657 as template.

Twenty of the 31 amino acid substitutions identified in our cohort showed the highest probability of altering local protein structure and were therefore classified as 'probably damaging'. Of these 20 mutations, eight got the maximum score of one. Seven mutations were classified as 'possibly damaging' and four as 'benign'. The severity of the effect of the amino acid changes is independent from the affected domain although three of the four alternations classified as 'benign' are three of the four alterations affecting the asparagine (N) residue at position 466 (N466D, N466S, N466T). The fourth mutation affecting the same residue (N466K) is classified as 'probably damaging'.

All amino acid substitutions, their PolyPhen score and the predicted effect on the protein are summarised in Table 3.2; furthermore, examples of three Polyphen reports are shown in figure 1 of the Appendix.

DNA sequence change	amino acid change	Polyphen score	Polyphen prediction
c.172C>T	H58Y	0,925	possibly damaging
c.1025G>A	G342D	1	probably damaging
c.1144C>T	R382W	1	probably damaging
c.1145G>T	R382L	1	probably damaging
c.1145G>A	R382Q	1	probably damaging
c.1150T>A	F384L	0,993	probably damaging
c.1166C>T	T389I	0,997	probably damaging
c.1234A>G	T412A	0,789	possibly damaging
c.1268G>A	R423Q	0,997	probably damaging
c.1396 A>G	N466D	0,092	benign
c.1397A>G	N466S	0,000	benign
c.1397A>C	N466T	0,073	benign
c.1398C>G	N466K	0,606	possibly damaging
c.1407G>T	Q469H	0,998	probably damaging
c.1771A>G	K591E	0,020	benign
c.1843A>G	K615E	0,580	possibly damaging
c.1865C>T	T622I	1	probably damaging
c.1907C>A	S636Y	0,996	probably damaging
c.1909G>A	V637M	1	probably damaging
c.1909G>T	V637L	0,865	possibly damaging
c.1910T>C	V637A	0,974	probably damaging
c.1915C>T	P639S	0,988	probably damaging
c.1916C>G	P639R	0,999	probably damaging
c.1918T>A	Y640N	1	probably damaging
c.1970A>G	Y657C	1	probably damaging
c.2003C>T	S668F	0,996	probably damaging
c.2113T>C	Y705H	0,995	probably damaging
c.2117T>C	L706P	0,995	probably damaging
c.2124C>G	T708S	0,865	possibly damaging
c.2129T>C	F710C	0,997	probably damaging
c.2141C>G	T714A	0,956	possibly damaging

Table 3.2: Predicted impact of amino acid substitutions

Bioinformatic assessment of STAT3 protein missense changes and the prediction on the STAT3 function using the online prediction tool PolyPhen. Mutations classified as 'probably damaging are shaded in red, those classified as 'possibly damaging' in yellow, whereas mutation classified as 'benign' are shaded in green.

3.4 Discussion

In our cohort of 153 patients with suspected AD-HIES, we found 25 novel mutations in *STAT3* that had not been described at the time of discovery. Ten of the novel mutations affected the DNA-binding domain, eight the SH2 domain, six the transcription activation domain, and one the N-terminal domain (Table 3.1). Therefore, it appears necessary to sequence the entire *STAT3* gene to exclude possible mutations.

In all HIES patients with detected *STAT3* mutations, only one allele was affected, consistent with the observation that complete loss of *Stat3* leads to early embryonic death in Stat3 knock-out mice (Takeda et al., 1997).

Mutations in the promoter region are possible, but are unlikely to cause AD-HIES when present in heterozygosity because they will not exert a dominant-negative effect which seems necessary for the AD-HIES phenotype.

Although over 90% of mutations were located in the exons of *STAT3*, six mutations at the intron-exon boundaries were found. Five of these mutations caused an in-frame deletion of the 10 amino acids encoded by exon 12. The effect of the splice site mutation on exon 22 has been shown to be more complex: the splice site introduced by the mutation causes the 43 bp exon 22 to be skipped. In mutant variants of the two transcripts NM_139276 and NM_003150, this deletion would result in a frame shift and a premature stop-codon. In conjunction with the 50-bp shorter exon 23 of STAT3β, the deletion of exon 22 is predicted to produce a transcript that codes for a variant of STAT3α with an in-frame deletion of 31 amino acids.

PolyPhen-2 (Polymorphism Phenotypingv2 genetics.bwh.harvard.edu/pph2) is a free online tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein. The PolyPhen analysis of the 31 missense mutations found suggested that 20 amino acid substitutions

identified in our cohort have the highest probability of altering local protein structure and were classified as probably damaging. Seven of the remaining 11 mutations were classified as possibly damaging, four as benign. Interestingly, three of the four alternations classified as benign are three of the four alterations affecting the asparagine (N) residue at position 466 (N466D, N466S, N466T). For one of these mutations (N466S) familial inheritance has been observed (Figure 3.14). This makes it likely that the mutation is disease causing although not classified as damaging. Moreover, this mutation has been shown to have severe impact on the functionality of STAT3 (see chapter 4). We therefore conclude that the PolyPhen classification is not always correct and that in this case the mutation has an impact on the function of the STAT3 protein.

The fourth mutation classified as benign is the substitution K591E.

Familial inheritance has been observed in nine cases. Five out of these nine are novel mutations that had not been described at the time of discovery. In all cases the inheritance of the mutation correlates perfectly with the affection status. The familial inheritance therefore might indicate that these mutations are disease causing although no functional data are available for all of them. In particular this is interesting for the mutation affecting the N-terminal domain since no other mutations in this domain have been reported.

In collaboration with the National Center for Biotechnology Information at the National Institutes of Health (NIH) in Bethesda (USA) we evaluated the validity of the NIH clinical scoring sheet in predicting *STAT3* mutations and sought for a correlation between the phenotype of the patients and the genotype.

On the basis of the clinical features of the patients no significant genotype/phenotype correlation was evident between patients with mutations in the DNA-binding, the SH2, the transcriptional activation- or the N-terminal domain, indicating that the location of the mutation has no infuence on the

resulting phenotype. Similarly, there was no clear difference in the clinical presentation between the cohort of patients with *STAT3* mutations and 71 patients without a demonstrable mutation (Woellner et al., 2010).

The same results were confirmed by other groups (Heimall et al., 2011; Renner et al., 2008).

The wide repertoire of mutations identified within the STAT3 gene represents a broad tool to perform structure/function studies on STAT3 and gives the opportunity to investigate fundamental aspects of STAT3 biology that are still unanswered.

The evidence that the STAT3 mutations are located in different areas of the protein known to exert distinct functions suggests that more than one molecular mechanism leads to the same clinical presentation. It has been shown that mutations in the DNA-binding domain cause a defective binding of STAT3 to the DNA (Minegishi et al., 2007) whereas mutations affecting the SH2 domain impair the activation-induced tyrosine phosphorylation of STAT3. The molecular mechanism by which heterozygous mutations in the N-terminal domain or the transcription activation domain of *STAT3* cause the AD-HIES phenotype remains unclear.

The N-terminal domain of STAT3 (amino acids 1-125) has been shown to be essential for the formation of unphosphorylated STAT3 dimers but not for assembly of tyrosine-phosphorylated STAT3 dimers.

STAT3 harbouring a monomeric N-terminal deletion becomes phosphorylated and dimerizes in response to interleukin-6 stimulation but, surprisingly, does not accumulate in the nucleus. This highlights the importance of the N-terminal domain in the formation of unphosphorylated STAT3 dimers and nuclear accumulation of STAT3 upon phosphorylation (Vogt et al., 2011). Whether the lack of nuclear accumulation of STAT3 in the nucleus represents the molecular

mechanism leading to the AD-HIES phenotype in the two patients, carrying the mutation in the N-terminus of STAT3 should be investigated.

Seventy-one patients did not show any mutation in the coding regions of *STAT3* or their flanking intronic sequences. During the course of this study it became clear that homozygous mutations of the dedicator of cytokinesis 8 (DOCK8) gene are responsible for the autosomal recessive form of HIES (Engelhardt et al., 2009; Zhang et al., 2009). Four patients out of our cohort of 71 *STAT3* wild-type (*STAT3*wt) HIES patients were afterwards found to carry *DOCK8* mutations. Three of the four patients were shown to have big deletions of more than 20 exons (UPN81 ex3_26del, UPN82 ex2_24del, UPN21 ex18_44del). The fourth patient (UPN83) carries a smaller deletion of exons 1 and 2. The clinical details of the four patients are summarized in Table 1 in the appendix.

In the following patients with anAD-HIES phenotype without proven STAT3 mutation will be referred to as type 1 HIES patients as no inheritance pattern has yet been identified in these patients.

Chapter 4 : Functional impact of *STAT3* mutations

4.1 Introduction

In the previous chapter we showed that more than 50% of patients referred to us with the clinical diagnosis of AD-HIES, carried a *STAT3* mutation. Although the mutations found mainly cluster in the DNA-binding or the SH2 domain, mutations were very heterogeneous and were located all over the gene. Many of the mutations we found were yet undescribed and their effect on the function of STAT3 unknown. We therefore sought for a test able to show the effect of the newly discovered mutations on the functionality of STAT3. Furthermore, because the mutations were spread over the whole gene, it appears necessary to sequence the entire *STAT3* gene to exclude any possible mutation, an expensive process because of the size of the gene. It therefore was desirable to find a test able to predict the existence of a mutation without the need of sequencing.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine exclusively signalling via STAT3. IL-10 is produced by several cell types including macrophages and regulatory T-cells and inhibits the synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF (Fiorentino et al., 1991; Moore et al., 2001).

The receptor for IL-10 consists of two alpha molecules (IL10R1) specific to the interleukin-10 receptor and two beta molecules (IL10R2). The assembly of this heterotetrameric receptor leads to the activation of the receptor-associated Janus tyrosine kinases, JAK1 and TYK2, resulting in the phosphorylation of STAT3 and the induction of STAT3-dependent genes (Glocker et al., 2009). It has been shown that the IL-10-mediated inhibition of LPS-induced TNF-α production is defective in patients carrying mutations in *STAT3* (Minegishi et al., 2007).

Another common feature of patients with AD-HIES are significantly reduced numbers of Th17 cells, a T cell subset characterised by expression of the lineage defining transcription factor RORγt and the cytokines IL-17 and IL-22 (Stockinger and Veldhoen, 2007). An important factor to drive human Th17 cell differentiation is the STAT3 activating cytokine IL-6 (Laurence and O'Shea, 2007). IL-6 signals through STAT3, as does IL-23, a cytokine important for sustaining Th17 cells; STAT3 signalling therefore plays a crucial role in the generation of Th17 cells. IL-21, another activator of STAT3, is both a product and inducer of Th17 cells, providing a critical autocrine feedback loop (Laurence and O'Shea, 2007).

Several groups have reported reduced numbers of circulating Th17 cells in AD-HIES patients with *STAT3* mutations (de Beaucoudrey et al., 2008; Jiao et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008).

Reduced Th17 cell numbers are thought to add most to the clinical phenotype of AD-HIES especially to the increased susceptibility to bacterial and fungal infections. Reports that haematopoietic stem cell transplantation (HSCT) may correct this deficit underline the relevance of Th17 cells in the pathogenesis of AD-HIES (Goussetis et al., 2010).

4.2 Questions

1) Do all *STAT3* mutations have the same functional consequences – independently of which domain they affect?
2) Do all mutations lead to reduced numbers of Th17 cells? Do patients with a clinical phenotype of AD-HIES but without mutations within *STAT3* (type 1 HIES patients) have reduced numbers of Th17 cells as well?
3) Can the application of an IL-10 suppression assay or the enumeration of IL-17 producing peripheral blood $CD4^+$ T-cells be used as potential functional tests for AD-HIES able to predict the presence of a STAT3 mutation?

4.3 Results

4.3.1 Functional consequences of *STAT3* mutations

To analyse the consequences of *STAT3* mutations on the functionality of the signalling pathway *in vitro*, we used a TNF-α release assay as previously described by Minegishi et al (Minegishi et al., 2007). We preincubated 10^6 monocytes/macrophages/mL of 12 patients and 11 healthy controls with 25ng/mL IL-10 for 1 hour and then stimulated with 50ng/mL LPS overnight. As IL-10 is a potent anti-inflammatory cytokine signalling via STAT3, the addition of IL-10 to the cells was expected to inhibit the production of TNF-α upon addition of LPS.

In healthy controls and a patient without *STAT3* mutation (*STAT3*wt), the addition of IL-10 reduced the TNF-α release by approximately 90%, whereas in a patient carrying a R382Q mutation in in the DNA-binding domain of *STAT3*, TNF-α release was reduced by only 40% (Figure 4.1).

To ensure better comparability of data from different healthy donors and patients, the impact of IL-10 on TNF-α release is shown as percentage of maximum TNF-α release on LPS stimulation as shown in Figure 4.1.

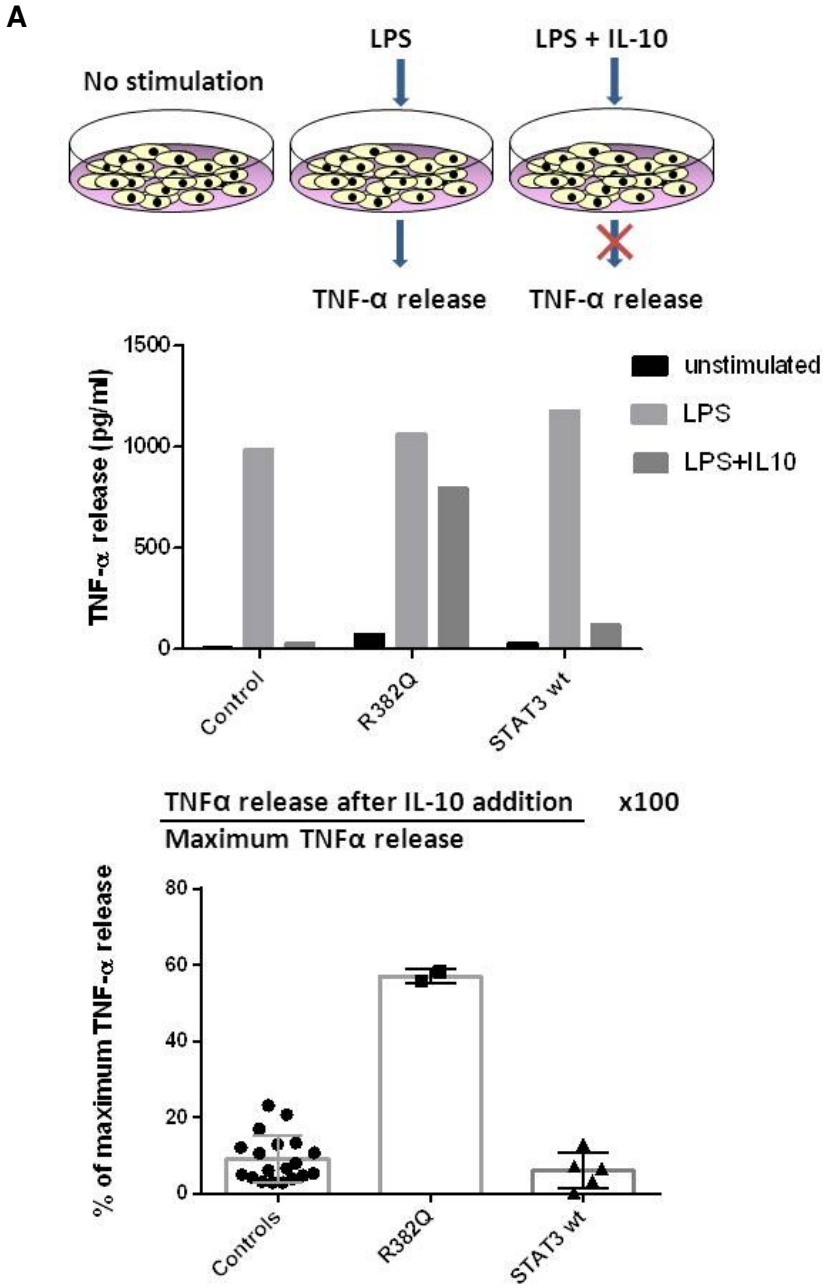


Figure 4.1: Schematic representation of the TNF-α release assay

(A) Cells were exposed overnight to LPS or LPS plus IL-10. The concentration of produced TNF-α in the culture supernatants was determined by ELISA. In healthy controls and a *STAT3*wt patient, addition of IL-10 almost completely reduced TNF-α release, whereas in the patient with the R382Q mutation in *STAT3* inhibition of LPS-induced TNF-α release is poor. (B) To ensure better comparability of data from different healthy donors and patients, the impact of IL-10 on TNF-α release is shown as percentage of maximum TNF-α release on LPS stimulation.

In our cohort of more than 150 patients with the clinical AD-HIES phenotype, we found 25 mutations in *STAT3* that have not been previously described. To assess the effects of these novel *STAT3* mutations, we carried out the TNF-α release assay as described above.

We tested eight novel mutations: one affecting the N-terminal domain (H58Y), three affecting the DNA-binding domain (c.1139+2insT, N466S and N466K), one affecting the SH2 domain (P639S) and two affecting the transcriptional activation domain (L706P and F710C). In addition we examined four already known mutations affecting the DNA-binding domain (c.1110-2A>G, R382Q, R382W and R382L) and 11 controls.

All eight novel mutations showed a reduction in IL-10-mediated down regulation of TNF-α secretion. Not all mutations however had the same effect, suggesting that some mutations may lead to STAT3 proteins with a hypomorphic function and a remaining activity, and therefore possibly to a milder phenotype. Furthermore, we experienced great variability in the effect of some mutations in different experiments, resulting in distinct standard deviations (Figure 4.2)

Additionally, we compared the effect of the eight novel mutations collectively with 11 healthy controls by a rank-sum test and reached significance ($P < .0001$).

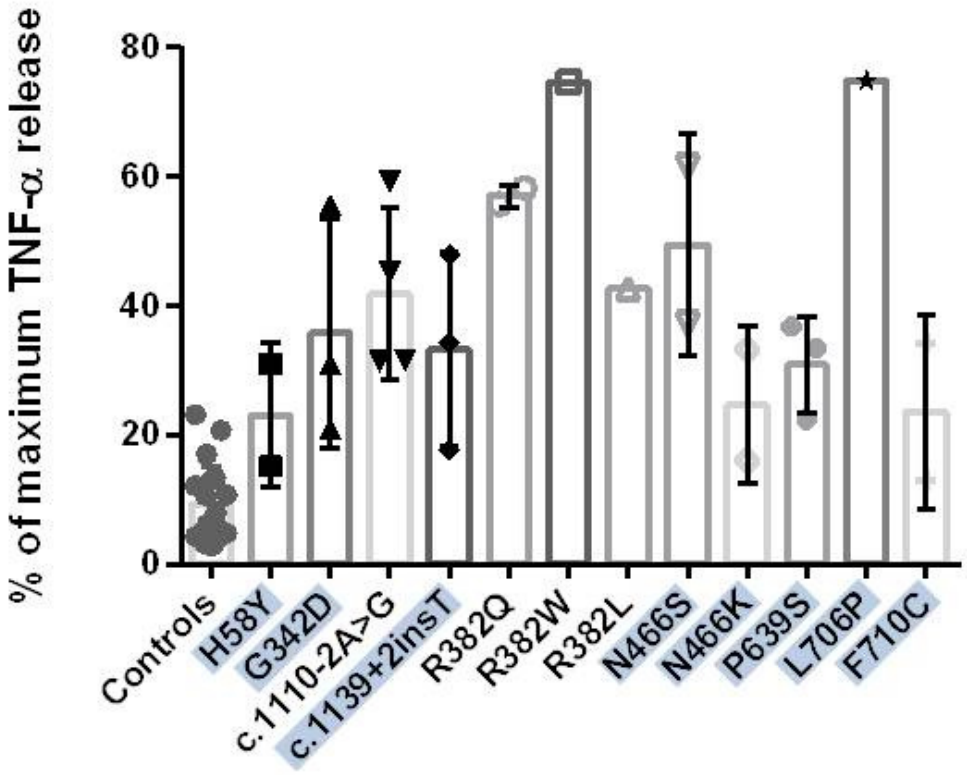


Figure 4.2: Inhibition of TNF-α release in LPS-activated macrophages by IL-10.

Inhibition of TNF-α release in LPS-activated macrophages by IL-10. Macrophages of 12 patients with mutant *STAT3*, 8 of whom had novel mutations, and 11 healthy controls were pre-treated with IL-10 and then stimulated with LPS. Supernatants were examined for the presence of TNF-α by ELISA. To ensure better comparability of data from different healthy donors, the impact of IL-10 on TNF-α release is shown as percentage of maximum TNF-α release on LPS stimulation. The eight undescribed mutations are shaded in blue. Error bars represent standard deviations. Where possible results are representative of at least two independent experiments. Due to lack of additional cells the mutations R382W, R382L and L706P could be tested only once.

4.3.2 TNF-α ELISA as functional test for *STAT3* mutations

As the *STAT3* gene is a big gene consisting of 24 exons and because the mutations affecting *STAT3* are spread over the whole gene, we analysed the possibility to use the TNF-α ELISA as test able to predict the existence of a mutation without the need of sequencing.

A patient with suspected Dubowitz syndrome, characterized by growth retardation, microcephaly and eczema, with autosomal recessive inheritance but unclear genetic basis was referred to us together with her brother and the parents. The patient had a long history of *S. aureus* infections affecting different organs and presented aged 31 with an NIH-HIES score of 77 points. When the patient presented with an episode of meningitis and cerebral abscesses, a clinical diagnosis of the putative AD-HIES in coexistence with a Dubowitz-like syndrome was made (Beitzke et al., 2011).

The cells of the patient (UPN185) and those of her parents, her brother and one healthy control were stimulated with LPS and IL-10 as described above.

In contrast to the healthy control, the parents and her brother, the patient's cells showed significantly impaired IL-10-mediated suppression of TNF-α release, indicating a failure of STAT3 signalling. Subsequent *STAT3* sequencing revealed a heterozygous missense mutation in exon 13 leading to the amino acid exchange R382W in the patient, whereas both parents and the brother showed a wild-type sequence (Figure 4.3). Therefore, in this case, we were able to correctly predict the existence of a *STAT3* mutation in the patient and no mutation in the patient’s family members.

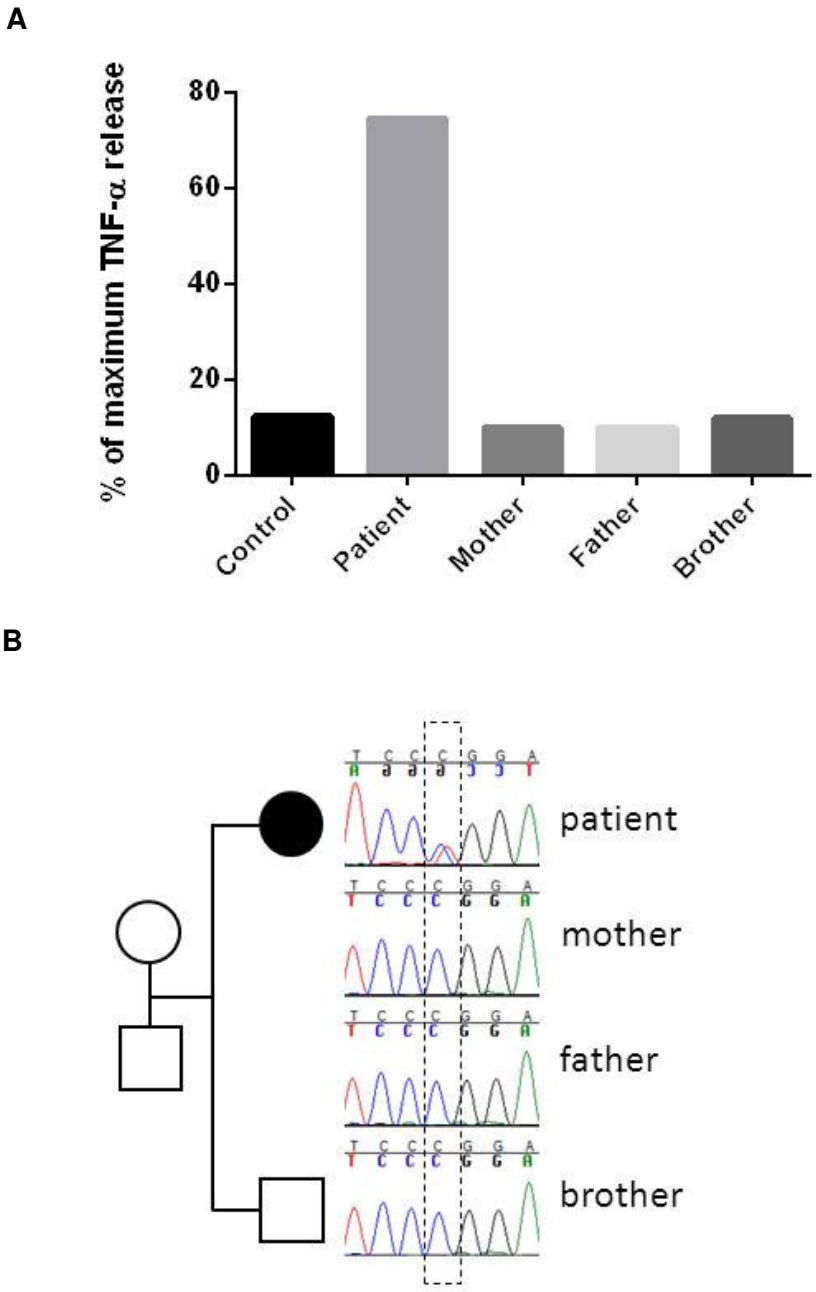


Figure 4.3: TNF-α ELISA as functional test for *STAT3* mutations

(A) Cells of the patient (UPN185), her parents, her brother and a healthy control were exposed overnight to LPS or LPS plus IL-10. The concentration of produced TNF-α in the culture supernatants was determined by ELISA. The parents and the brother of the patient, as the healthy donor, show almost complete reduction of TNF-α release upon IL-10 treatment indicating unimpaired STAT3 signalling. The patients' cells show poor inhibition of LPS-induced TNF-α release. (B) Pedigree and the relative chromatograms of the patient and her family. The patient carries the sequence change 1144C>T leading to amino acid change R382W. Both parents and the brother show wild-type sequence therefore confirming the results of the TNF-α release assay.

4.3.3 IL-17 and IFN-γ production by CD4$^+$ T cells in AD-HIES

The induction of Th17 cells after stimulation with either the superantigen Staphylococcus enterotoxin B (SEB) or the mitogen Phorbol 12-myristate 13-acetate (PMA) and Ionomycin has been reported to be impaired in patients with AD-HIES carrying *STAT3* mutations (de Beaucoudrey et al., 2008; Jiao et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008). We analysed the level of Th17 cells among the PBMCs in 37 out of our 153 patients with AD-HIES and 24 control subjects. Nineteen of these patients had mutations in *STAT3*, 18 had not. Cells were stimulated with SEB over night as described in the methods-section.

Figure 4.4 shows the stimulation and the gating of a representative healthy control. Lymphocytes were first gated for CD4$^+$ and CD45RO$^+$ memory T cells and then on intracellular stained IL-17 and IFN-γ. The unstimulated control cells did neither produce IFN-γ nor IL-17. In contrast, stimulated PBMCs of the healthy controls showed 11.7% of IFN-γ-producing and 1.73% of IL-17-producing memory T cells.

Seventeen of the 19 patients harbouring mutations in *STAT3* had less than 0.5% of IL-17-producing CD4$^+$ T cells. In contrast, all but two of our healthy donors had a frequency of more than 0.6%.

Figure 4.5 shows representative flow cytometry plots of one healthy control, one patient carrying a mutation in the DNA-binding domain (R382W), one patient with a mutation in the SH2 domain (V637M) and one *STAT3*wt type 1 HIES patient. It is clearly visible that patients carrying a *STAT3* mutation had an impaired number of circulating Th17 cells.

The 18 patients without mutations in *STAT3* had significantly more IL-17-producing T cells than the STAT3-deficient patients ($P<0.0001$), but significantly fewer than the healthy controls ($P=0.0094$); with less than 0.5%

only four of these 18 patients had a low percentage of IL-17-producing $CD4^+CD45RO^+$ T cells (Figure 4.6 and Table 4.1).

We measured the frequency of SEB-induced IFN-γ-producing $CD4^+$ T cells from PBMCs of the same set of subjects (Figure 4.5 and 4.6). Interestingly, the type 1 HIES patients without *STAT3* mutations had significantly fewer IFN-γ-producing $CD4^+$ T cells than had healthy controls ($P<0.0001$). Of the 18 patients lacking *STAT3* mutations, only two had >5% IFN-γ-producing $CD4^+$ T cells. Patients with AD-HIES harbouring *STAT3* mutations also had significantly lower percentages of IFN-γ-producing $CD4^+$ T cells than healthy controls ($P=0.047$) but significantly higher percentages than patients without *STAT3* mutation ($P=0.0002$).

Although the lack of Th17 cells or IL-17 has been made responsible for the occurrence of *Candida albicans* infections among our patients we did not find any correlation between the lack of Th17 cells and the incidence of *Candida* infections (Table 4.1 and Figure 4.7).

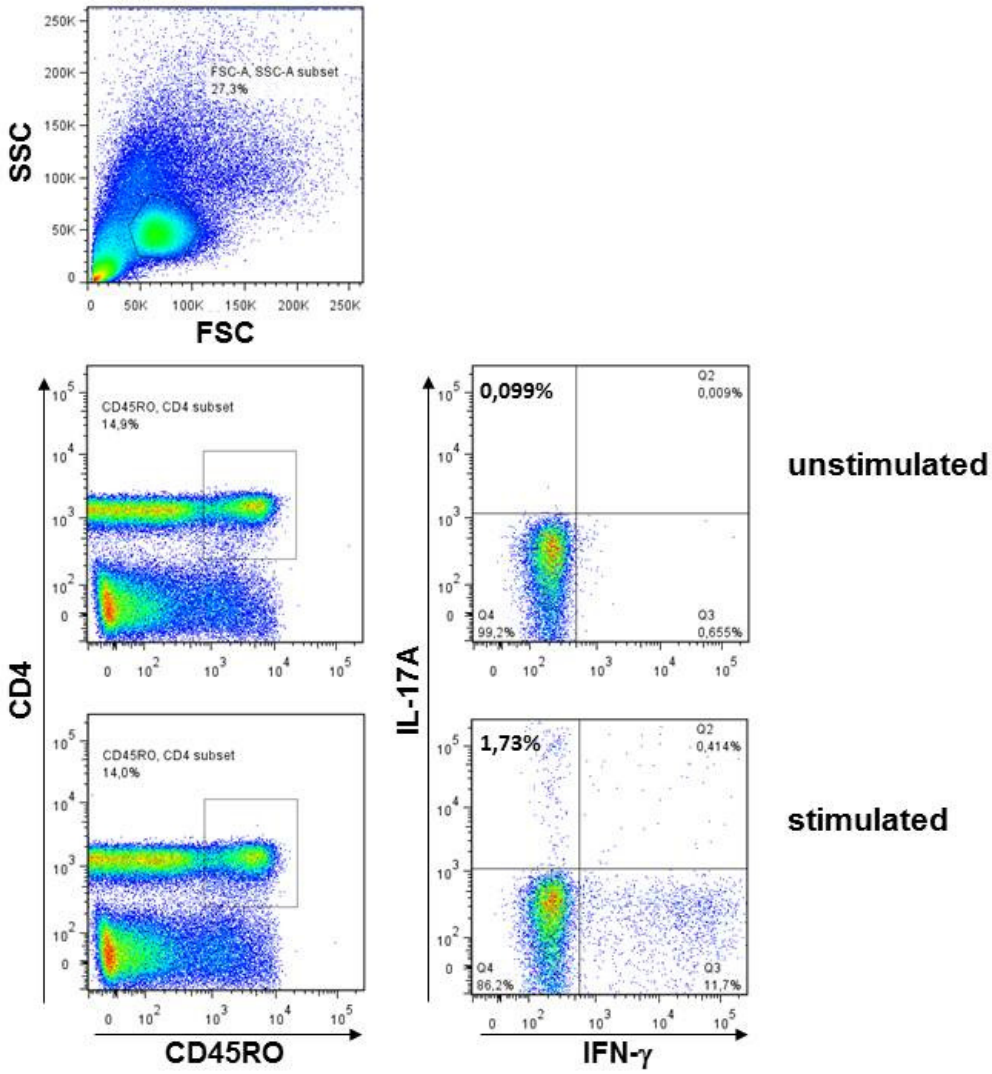


Figure 4.4: IL-17 and IFN-γ production from PBMCs of a healthy control

Flow cytometry analysis of $CD4^+CD45RO^+$ memory T cells from healthy control PBMCs stained intracellular for IL-17 and IFN-γ. Cells were stained either unstimulated or upon stimulation with SEB for 16 hours, Brefeldin A was added after one hour stimulation.

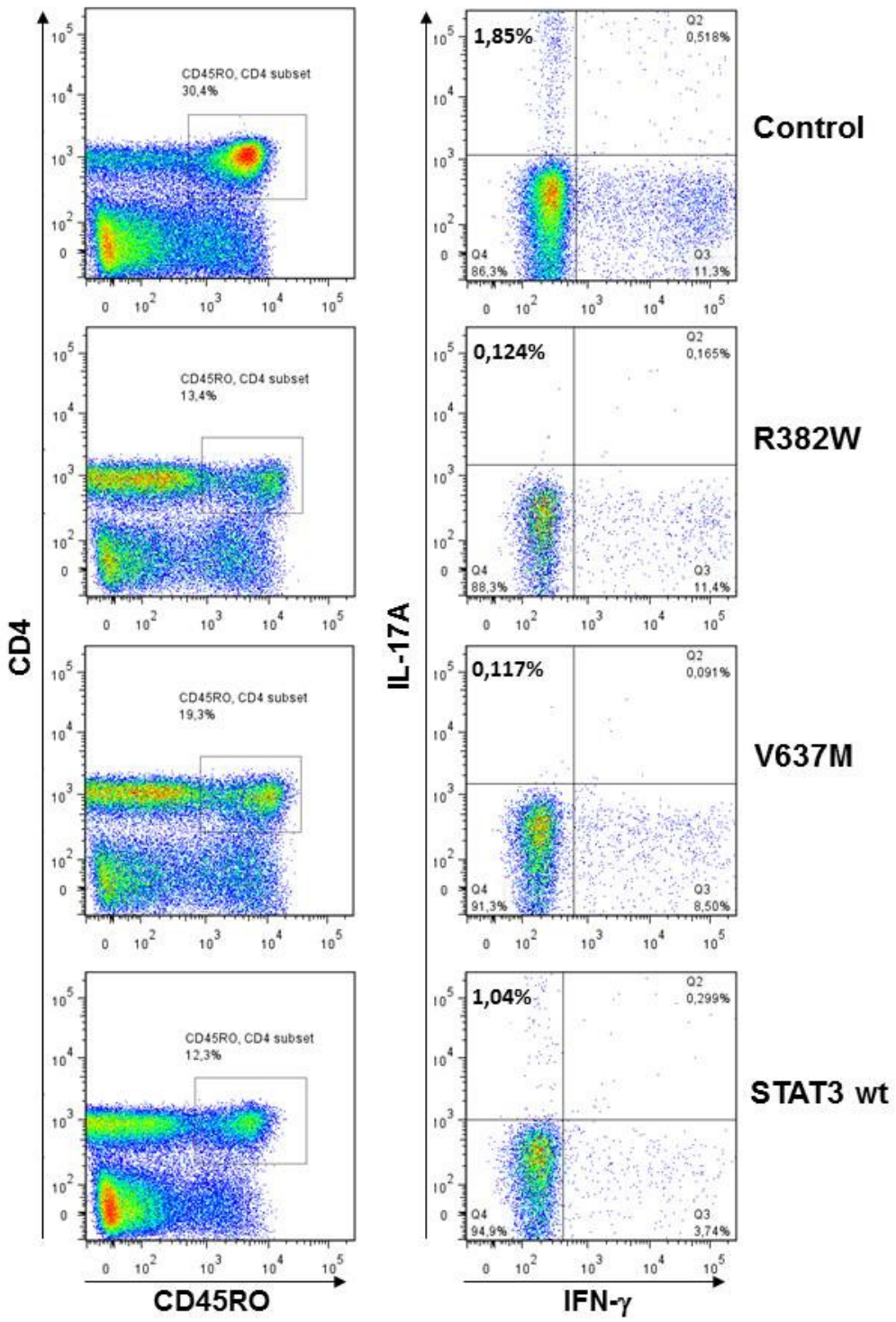


Figure 4.5: IL-17 and IFN-γ production from PBMCs HIES patients

Flow cytometry analysis of $CD4^+CD45RO^+$ memory T cells from one healthy control one AD-HIES patient carrying the STAT3 mutation R382W, one patient with the mutation V637M and one STAT3wt type 1 HIES patient stained intracellular for IL-17 and IFN-γ. Prior to staining cells were stimulated with SEB for 16 hours, Brefeldin A was added after one hour stimulation. Patients carrying *STAT3* mutations have significantly reduced numbers of Th17 cells when compared to the healthy control and the *STAT3*wt type 1 HIES patient.

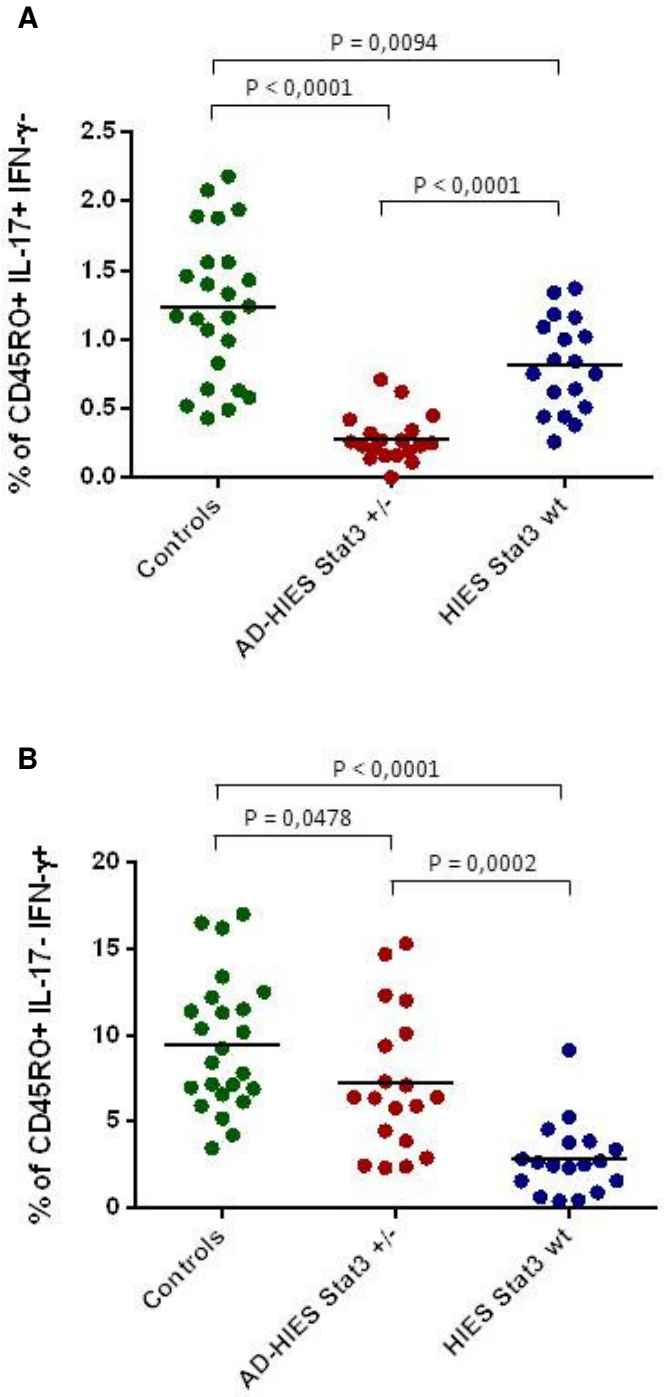


Figure 4.6: Percentage of IL-17 and IFN-γ expressing CD4⁺ T cells

Percentages of IL-17 (A) and IFN-γ (B) expressing $CD4^+CD45RO^+$ memory T cells, determined by intracellular cytokine expression after overnight stimulation with SEB. Each symbol represents the value from an individual donor or patient. Statistical significance was determined with a Wilcoxon rank-sum test. P values are 2-sided. Median values are shown as horizontal bars. wt, wild-type.

UPN	DNA sequence change	Predicted amino acid change	Domain	NIH score*	% of CD45RO$^+$ IL17$^+$ IFN-γ$^-$	% of CD45RO$^+$ IL17$^-$ IFN-γ$^+$	Candidiasis
110	c.1025G>A	G342D	DNA-binding	79	0.27	12.3	yes
114	c.1110−2A>G	D371_G380del	DNA-binding	70	0.26	5.9	yes
86	c.1110−1G>T	D371_G380del	DNA-binding	83	0.16	6.36	yes
17	c.1139+1G>T	D371_G380del	DNA-binding	65	0.34	2.34	yes
108	c.1119+2insT	D371_G380del	DNA-binding	54	0.19	4.47	yes
15	c.1144C>T	R382W	DNA-binding	76	0.23	15.3	yes
19	c.1144C>T	R382W	DNA-binding	63	0.62	7.11	yes
126	c.1144C>T	R382W	DNA-binding	58	0.16	7.32	yes
128	c.1144C>T	R382W	DNA-binding	29	0.27	2.91	yes
183	c.1145 G>A	R382Q	DNA-binding	nd	0.14	2.45	nd
98	c.1145 G>T	R382L	DNA-binding	45	0.11	14.7	yes
118	c.1268G>A	R423Q	DNA-binding	59	0.23	5.78	no
71	c.1907C>A	S636Y	SH2	71	0.32	2.4	yes
16	c.1909G>A	V637M	SH2	68	0.25	10.1	yes
115	c.1909G>A	V637M	SH2	56	0.71	6.4	nd
125	c.1909G>A	V637M	SH2	58	0.21	9.39	no
49	c.1915 T>C	P639S	SH2	55	0	3.89	yes
18	c.2129T>C	F710C	Transactivation	58	0.42	12	yes
116	c.2141C>G	T714A	Transactivation	63	0.45	6.42	yes
1	no mutation			72	0.38	2.65	yes
4	no mutation			60	0.84	9.15	yes
9	no mutation			49	0.44	1.59	yes
10	no mutation			29	0.26	0.65	no
14	no mutation			47	0.44	2.73	no
20	no mutation			32	1.16	3.87	no
22	no mutation			43	0.62	2.85	yes
54	no mutation			29	0.75	2.46	yes
55	no mutation			26	0.51	3.39	no
69	no mutation			61	1.09	5.27	yes
97	no mutation			39	1.18	2.33	yes
113	no mutation			39	1.02	0.46	no
123	no mutation			30	1.34	0.91	no
124	no mutation			22	1.37	0.42	nd
127	no mutation			49	1	4.58	yes
139	no mutation			40	0.64	3.8	yes
163	no mutation			nd	0.85	2.55	nd
184	no mutation			nd	0.75	1.58	nd

Table 4.1: Percentages of IL-17 and IFN-γ expressing CD4$^+$ T cells

Patients with less than 0.5% CD4$^+$CD45RO$^+$IL17$^+$IFN-γ$^-$ or 5% CD4$^+$CD45RO$^+$IL17$^-$ IFN-γ$^+$ cells are shaded in blue. Patients positive for Candida infections are shaded in purple. Undescribed mutations are shaded in grey.
UPN, unique patient number * scoring system described in Grimbacher et al. 1999

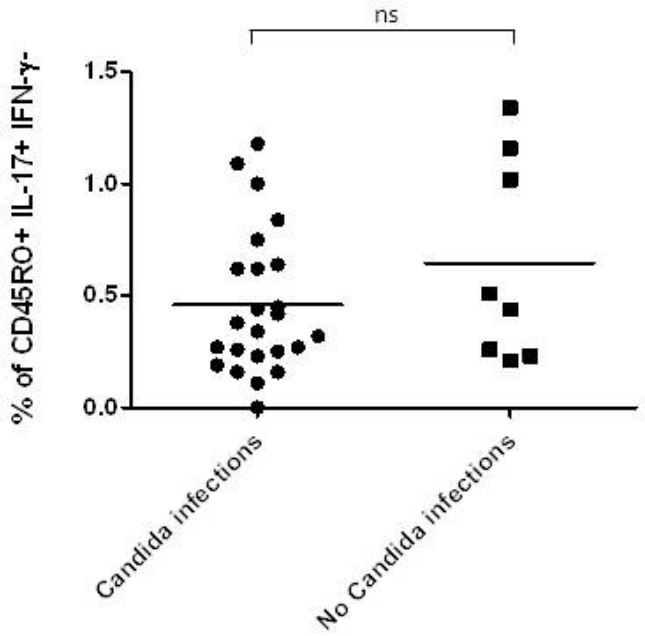


Figure 4.7: Correlation between numbers of IL-17 expressing $CD4^+CD45RO^+$ T cells and the presence of *Candida albicans* infections

Numbers of IL-17 expressing $CD4^+CD45RO^+$ memory T cells determined by intracellular cytokine expression after overnight stimulation with SEB of AD-HIES patients with and without *Candida albicans* infections. Each symbol represents the value from an individual donor or patient. Statistical significance was determined with a Wilcoxon rank-sum test. Median values are shown as horizontal bars.ns, non-significant.

4.3.4 Th17 cells in a bone marrow transplanted patient

One patient of our cohort (UPN56) who carried the mutation R382W underwent haematopoietic stem cell transplantation (HSCT) in 2005 at the age of 13. The patient had a long history of recurrent chest infections with longstanding established bronchiectasis and despite prophylactic antibiotics and intravenous immunoglobulin, the patient's condition continued to deteriorate.

After transplantation the patient had good immune reconstitution and far less infections. As expected, the already existing bronchiectasis did not improve following transplantation, but further lung infections and lung damage were prevented.

We measured the levels of circulating IL-17-producing $CD3^+$ and $CD45RO^+$ memory T cells in the patient's PBMCs before and after the HSCT and compared them with the levels of Th17 cells of a healthy control and a patient with a *STAT3* mutation (V637M). Prior to staining, the cells were stimulated with PMA/Ionomycin for five hours as described above.

Before HSCT the patient had 0,3% of circulating IL-17 and 42,8% of IFN-γ-producing $CD3^+CD45RO^+$ T cells. In contrast, after the HSCT the number of circulating Th17 cells raised to 1,16%, comparably to the healthy control whereas the number of IFN-γ-producing cells decreased. The *STAT3* mutated patient had 0,2% of IL-17-producing and 51% of IFN-γ-producing cells.

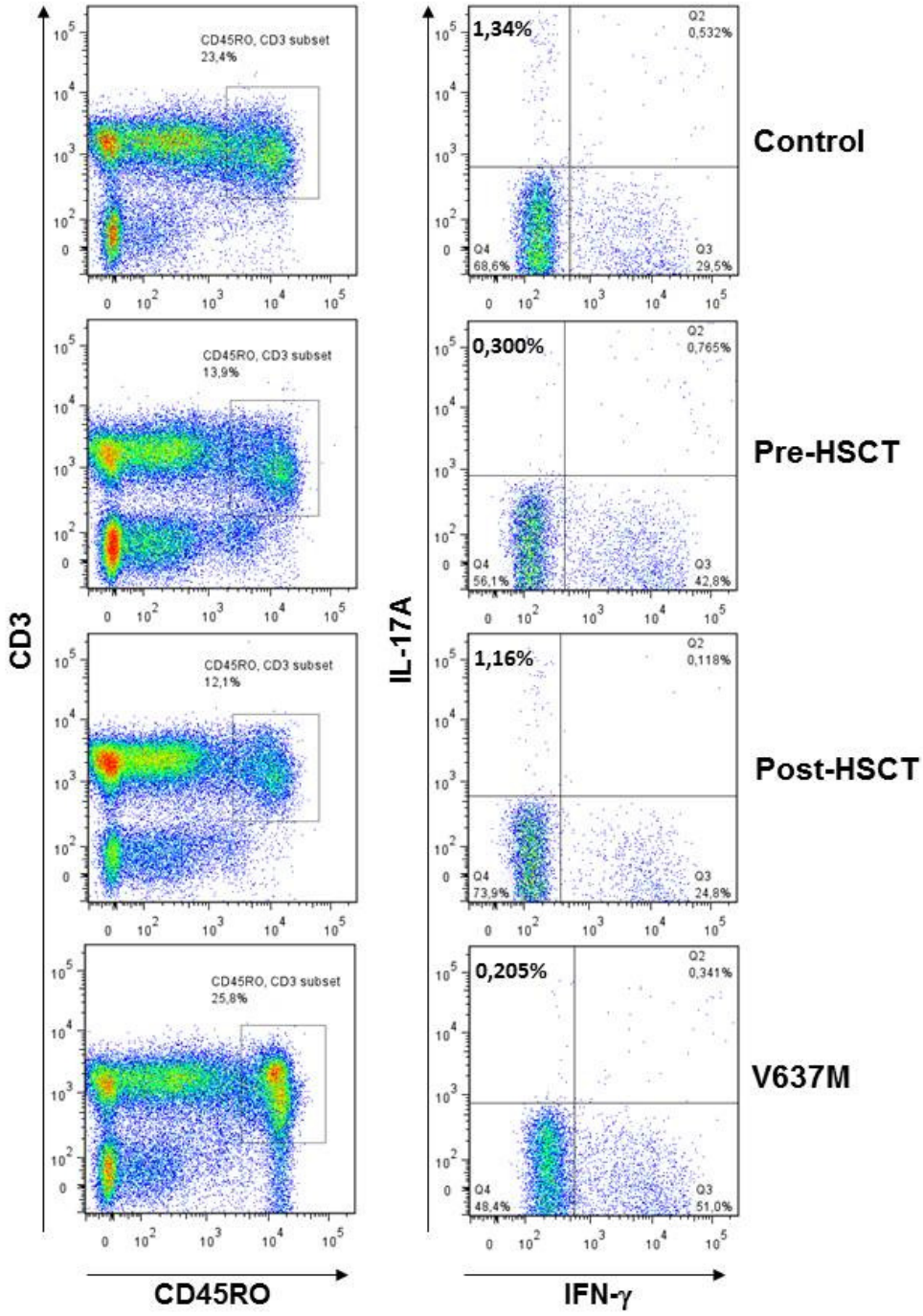


Figure 4.8: Th17 cells of bone marrow transplanted patient

Flow cytometry analysis of $CD3^+CD45RO^+$ memory T cells from one healthy control, one patient with the mutation V637M and the bone marrow transplanted *STAT3* mutated AD-HIES patient stained intracellular for IL-17 and IFN-γ. The cells of the patient were stained before and after bone marrow transplantation. Prior to staining cells were stimulated with PMA/Ionomycin for five hours, Brefeldin A was added after one hour stimulation**.** After HSCT the percentage of IL-17-producing cells is increased and comparable to the healthy control.

4.4 Discussion

We showed that all eight novel mutations we identified in *STAT3* impaired the IL-10-mediated inhibition of TNF-α in LPS challenged patients' macrophages indicating failures in the IL-10/Janus kinase/STAT3 pathway in the patients' cells. Compared with the most prevalent mutation affecting the DNA-binding domain of STAT3 (R382Q) this effect however was less prominent in some of the novel mutations, in particular the four mutations H58Y, N466K, P639S and F710C. This result could reflect the fact that these mutations have a different impact on the retained functionality of the STAT3 dimerization, nuclear translocation, or transcriptional activation. The variability of the remaining activity of the STAT3 protein may possibly lead to the observed diversity of phenotypes.

Moreover, we found great variability in TNF-α release for some *STAT3* mutations. Although 'classic' *STAT3* mutations lead to meaningful test results, (as shown for the family of patient UPN185) a remarkablevariability of results, leading to distinct standard deviations was observed. The differences in response to LPS stimulation at different time points could be due to various factors including lifestyle, the affection status and the medication of the patient at the time of blood-drawing. Moreover, most of the blood samples analyzed were sent to us from elsewhere and have therefore travelled for different periods of time. It is likely that all these variables influence the viability and responsiveness of the cells. As the variables mentioned are not eliminable, we decided that this assay is unsuitable as screening method to identify patients with *STAT3* mutations.

To assess whether the variability of the test results is due to different responsiveness of cells the analysis of a related pathway in which STAT3 is

not involved (like STAT1 signalling pathway) could have been performed in parallel.

There are other options to test the functionality of STAT3: The formation of phosphorylated STAT3 dimers in *STAT3* mutated patients is controversially discussed in the literature. Renner et al. showed that mutations in the SH2 domain but not those in the DNA-binding domain affect activation-induced tyrosine phosphorylation of STAT3 whereas Al-Khatib et al. found impaired pSTAT3 formation in patients with mutations affecting the SH2 as well as those in the DNA binding domain (Al Khatib et al., 2009; Renner et al., 2008).

The quantification of changes in the expression of STAT3 target genes such as *SOCS3* or *RORγt* by real-time PCR methods upon stimulation of patients' cells may be another possibility to predict a putative mutation in *STAT3*.

STAT3 mutations leading to the AD-HIES phonotype are heterogeneous and display a variable functional deficit. They all are dominant-negative with a suspected residual function of 25%. Since, it is difficult to set up a specific test capable of predicting all mutations.

We confirmed that patients with AD-HIES carrying *STAT3* mutations had significantly reduced numbers of IL-17-producing $CD4^+CD45RO^+$ T cells. Based on these data, we suggest that Th17 cells may be used as an additional marker to distinguish HIES patients with *STAT3* mutations from patients without *STAT3* mutations. Most patients without *STAT3* mutations had a striking reduction of IFN-γ-producing $CD4^+CD45RO^+$ T cells. This cytokine imbalance has been previously described in AD-HIES and may be explained by an intrinsic T-cell defect (Netea et al., 2005). In our cohort of *STAT3*wt type 1 HIES patients, only four of 18 had less than 0.5% IL-17-producing T cells. These three patients may have defects in other proteins involved in STAT3 signalling or in other pathways involved in the differentiation of Th17 cells.

The lack of Th17 cells or IL-17 producing cells has been made responsible for the occurrence of *Candida albicans* infections both in AD-HIES and in other defects leading to increased susceptibility to fungal infections. Nevertheless, among our patients were not able to identify any correlation between the lack of Th17 cells and the incidence of *Candida* infections.

One of the *STAT3* mutated AD-HIES patients of our cohort underwent haematopoietic stem cell transplantation in 2005 at the age of 13. The bone marrow transplant has been carried out with the expectation to cure the underlying immunologic defect. Indeed the patient has drastically improved, he suffered from less infections and the progressive lung destruction has been stalled. We showed that the number of circulating Th17 cells was restored after transplantation, indicating the importance of Th17 cells in the pathogenesis of AD-HIES with *STAT3* mutations. Allogeneic HSCT for patients with AD-HIES presenting with progressive lung disease might therefore considered.

Chapter 5 : Hunt for the genetic defect of *STAT3*wt type 1 HIES patients

5.1 Introduction

Seventy-one patients out of our cohort of 153 AD-HIES patients did not have any mutation in the coding regions of *STAT3* or the exon flanking intronic sequences. To rule out so far unknown splice site variants, the cDNA of 25 of these *STAT3* wild type patients was sequenced, but no mutation or skipped exon was detected.

As the type 1 HIES patients did not show any obvious difference with regards to the clinical presentation when compared with patients with proven STAT3 mutations we hypothesized that these patients may either carry (i) a mutation in the promoter of *STAT3* leading to a not yet described, apparent, disease associated haploinsufficiency; or (ii) a mutation in otherSTAT3 dependent pathways.

As described previously- STAT3 plays a central role in signal transduction induced by a broad range of cytokines, including IL-6, IL-10, IL-11, IL-17, IL-21 and IL-22.

In 2008 a Haitian boy has been identified with neutralizing anti-IL-6 autoantibodies: He suffered from two episodes of unusually severe cellulites and subcutaneous abscesses caused by *Staphylococcus aureus* (Puel et al., 2008). Since recurrent staphylococcal skin disease is a hallmark of HIES in both patients with mutations in STAT3 and without we decided to further focus on the IL-6 signalling pathway.

IL-6 has both pro-inflammatory and anti-inflammatory properties and is probably one of the best-studied cytokines using the STAT3 pathway. IL-6 is secreted by a variety of cells including T cells, B cells, macrophages, keratinocytes and fibroblasts. The biological activities of IL-6 include the regulation of immune response, inflammation, and haematopoiesis.

Additionally IL-6 is one of the most important mediators of fever and of the acute phase response (Mihara et al., 2012).

IL-6 signals through the IL-6 receptor, which consists of the ligand-binding IL-6RA chain (IL-6 receptor) and the signal-transducing component, the glycoprotein 130 (gp130). Whereas IL-6RA is specific to the IL-6 receptor, gp130 is needed for several other cytokines including IL-11, IL-27, leukemia inhibitory factor (LIF), ciliary inhibitory factor (CNTF), oncostatin M (OSM), and cardiotropin-1 (CT-1) (Taga and Kishimoto, 1997).

In contrast to gp130 which is ubiquitously expressed;, IL-6RA is primarily expressed on haemopoietic cells and hepatocytes. The binding of IL-6 to the membrane-bound IL-6RA leads to homodimerization of gp130 and formation of a functional receptor complex consisting of IL-6, IL-6RA and gp130 (Scheller et al., 2011).

Apart from the membrane bound form, a soluble form of IL-6RA (sIL-6RA) exists. Soluble IL-6RA arises from specific cleavage of membrane bound IL-6RA by endogenous enzymes, can also bind IL-6 and then form a complex with gp130. Many cells, which do not express membrane bound IL-6RA can respond to IL-6 in the presence of this agonistic sIL-6RA (Rose-John and Neurath, 2004).

After binding of IL-6 to its receptor the activation of the receptor associated tyrosine kinases of the JAK family, JAK1, JAK2 and TYK2 and subsequently of STAT3 is initiated. Figure 5.1 shows the components of the IL-6 receptor.

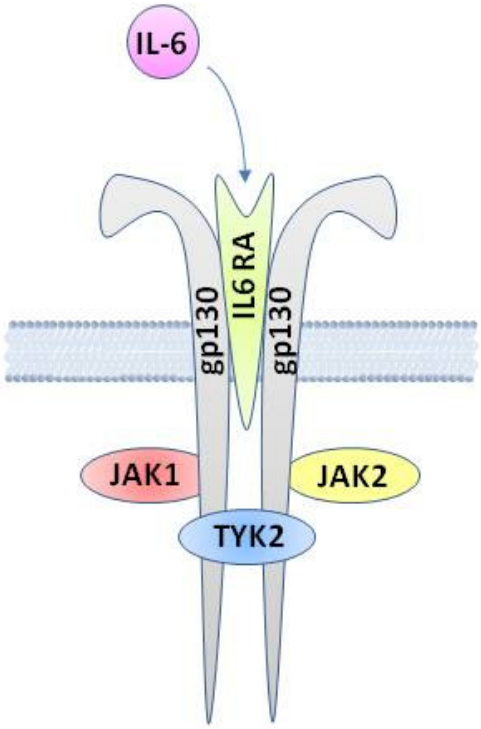


Figure 5.1: Components of the IL-6 receptor

Schematic representation of IL-6 and the IL-6 receptor. The receptor consists of the ligand-binding IL-6RA chain and the signal-transducing component, the glycoprotein 130 (gp130). The binding of IL-6 to the membrane-bound IL-6RA leads to homodimerization of gp130 and the formation of a functional receptor and to the activation of the receptor associated tyrosine kinases of the JAK family, JAK1, JAK2 and TYK2 and subsequently of STAT3.

5.2 Questions

1) Do patients with the clinical presentation of AD-HIES but without mutations in the coding regions of *STAT3* –or type 1 HIES patients carry mutations in the *STAT3* promoter or do they have a functional impairment of STAT3?
2) Do *STAT3*wt type 1 HIES patients carry mutations in one of the STAT3 related signalling pathways e.g. the IL-6 signalling pathway?
3) Or is the transition between the AD-HIES and AR-HIES phenotype more fluent and do *STAT3*wt patients have less severe mutations in DOCK8?
4) Is there any difference in the cytokine profile of cells of *STAT3*wt type 1 HIES patients after stimulation when compared to cells of *STAT3* mutant patients and healthy individuals?

5.3 Results

5.3.1 Patients from a geographically confined area

For further analysis of the IL-6/STAT3 signalling pathway five out of the 71 *STAT3*wt patients were chosen. These five patients all originated from Sardinia. Research suggests that Sardinians have a peculiar and differentiated genetic structure. Even though many groups of different origins arrived in Sardinia throughout history, Sardinia was isolated for a long time and therefore the Sardinian population has turned out to be an example of genetic isolation among Italian, European and Mediterranean populations (Calò et al., 2008). Because of the alleged peculiar genetic structure of the Sardinians we hypothesized that these five *STAT3*wt type 1 HIES patients may carry the same genetic aberration.

All five patients (four male and one female aged between eight and 21 years) presented with a typical clinical AD-HIES phenotype with a high NIH score, ranging between 43 and 72. To our knowledge the patients were neither related to each other nor descendants of consanguinous marriages. All patients had elevated serum IgE levels of more than 2000 IU/mL (up to a maximum value of 92,100 IU/mL), numerous skin abscesses, highly elevated numbers of eosinophil granulocytes in the peripheral blood (> 800/µL), severe eczema, repeated upper respiratory tract infections and candidiasis. All five presented with non-immunological features of AD-HIES, too. They had characteristic faces with an increased nasal width and high palate; three of them had scoliosis and had repeated pathological fractures due to minor trauma. Interestingly four of the five patients had experienced failure to shed three or even more deciduous teeth.

Only two of the patients had pneumonia and interestingly none of them showed any parenchymal lung abnormalities such as bronchiectasis or pneumatoceles which are typical for AD-HIES.

Two patients suffered from severe food allergies and asthma. Detailed information on the patient's phenotype is summarised in Table 5.1.

Whilst four patients were sporadic, one had a suspected family history (UPN1). The uncle of that patient died for unknown reasons in early childhood after an infection and the father of the patient was himself prone to illness (Figure 5.2).

Beside the AD-HIES phenotype this patient had a history of severe chronic diarrhoea, anaemia, and Herpes simplex virus infection during the first years of life.

UPN 1 21 y/o NIH score 72 4 abscesses, >3 pneumonia, >800 eosinophils/µl, newborn rash, eczema, >6 sinusitis/otitis, candidiasis, >3 retained primary teeth, scoliosis, fractures, characteristic face, increased nose width and high palate **Max IgE: 92,100 IU/mL** ➢ NO parenchymal lung abnormalities ➢ Allergies and asthma ➢ Severe chronic diarrhoea and anaemia in the first 4 years ➢ Herpes simples infection
UPN 4 19 y/o NIH score 60 3-4 abscesses, >800 eosinophils/µl, severe eczema, 3 sinusitis/otitis, candidiasis, >3 retained primary teeth, scoliosis, fractures, hyperextensibility, characteristic face, increased nose width, high palate and midline anomaly **Max IgE: 16,200 IU/mL** ➢ NO parenchymal lung abnormalities ➢ Allergies and asthma ➢ Tongue cleft
UPN 22 21 y/o NIH score 43 1-2 abscesses, >800 eosinophils/µl, newborn rash, eczema, 4-6 sinusitis/otitis, candidiasis, >3 retained primary teeth, characteristic face. **Max IgE: 19,100 IU/mL** ➢ NO parenchymal lung abnormalities
UPN 69 18 y/o NIH score 61 3-4 abscesses, 1 pneumonia, >800 eosinophils/µl, newborn rash, eczema, 4-6 sinusitis/otitis, candidiasis, >3 retained primary teeth, scoliosis, fractures, characteristic face, increased nose width, high palate and midline anomaly **Max IgE: 16,200 IU/mL** ➢ NO parenchymal lung abnormalities
UPN 139 8 y/o NIH score 43 1-2 abscesses, other serious infections, >800 eosinophils/µl, severe eczema, 4-6 sinusitis/otitis, candidiasis, hyperextensibility, characteristic face, increased nose width, high palate. **Max IgE: 58,100 IU/mL** ➢ NO parenchymal lung abnormalities

Table 5.1: Clinical features of five selected patients.

Age, NIH score, clinical features and maximal serum IgE levels measured for the five *STAT3*wt patients form Sardinia that were further analyzed.
UPN, unique personal number. IU, International units

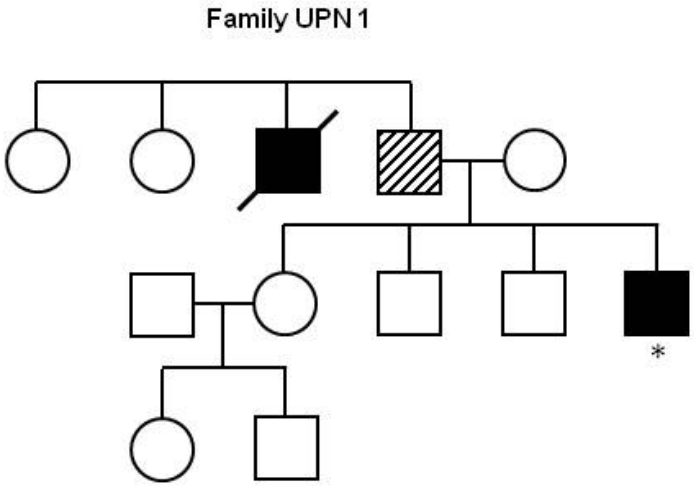


Figure 5.2: Pedigree of the family of patient UPN1 from Sardinia.

Squares indicate males; circles, females. Filled symbols, patients; slashes, deceased individuals. The shaded square indicates that the person is possibly affected. The proband is highlighted by an asterisk.

5.3.2 Sequencing of the *STAT3* promoter

Mutations in the promoter region of *STAT3* are possible, but are unlikely to cause AD-HIES when present in heterozygosity because they will not exert a dominant-negative effect, which seems necessary for the AD-HIES phenotype. Nevertheless, to exclude any undescribed effects of haploinsufficiency we sequenced the *STAT3* promoter regions in the five Sardinian patients. No sequence variation was found in any of the five patients in the sequence from -403bp upstream to +102bp within the *STAT3* gene, which possesses maximal promoter activity (Kato et al., 2000).

5.3.3 Testing for STAT3 function via TNF-α ELISA

To test the functionality of STAT3 in the five Sardinian patients, we measured the inhibition of TNF-α release in LPS-activated macrophages by IL-10. As described in the previous chapter, monocytes/macrophages of the five patients, two healthy controls and one patient with a previously reported R382Q *STAT3* mutation were pre-incubated with 25ng/mL IL-10 for one hour and then stimulated with 50ng/mL LPS overnight.

In the healthy control IL-10 reduced TNF-α release by approximately 90%, whereas in the R382Q patient, TNF-α release was reduced by only 40%. All five Sardinian patients, however, showed no reduction in IL-10-mediated down regulation of TNF-α secretion indicating that STAT3 is fully functional (Figure 5.3).

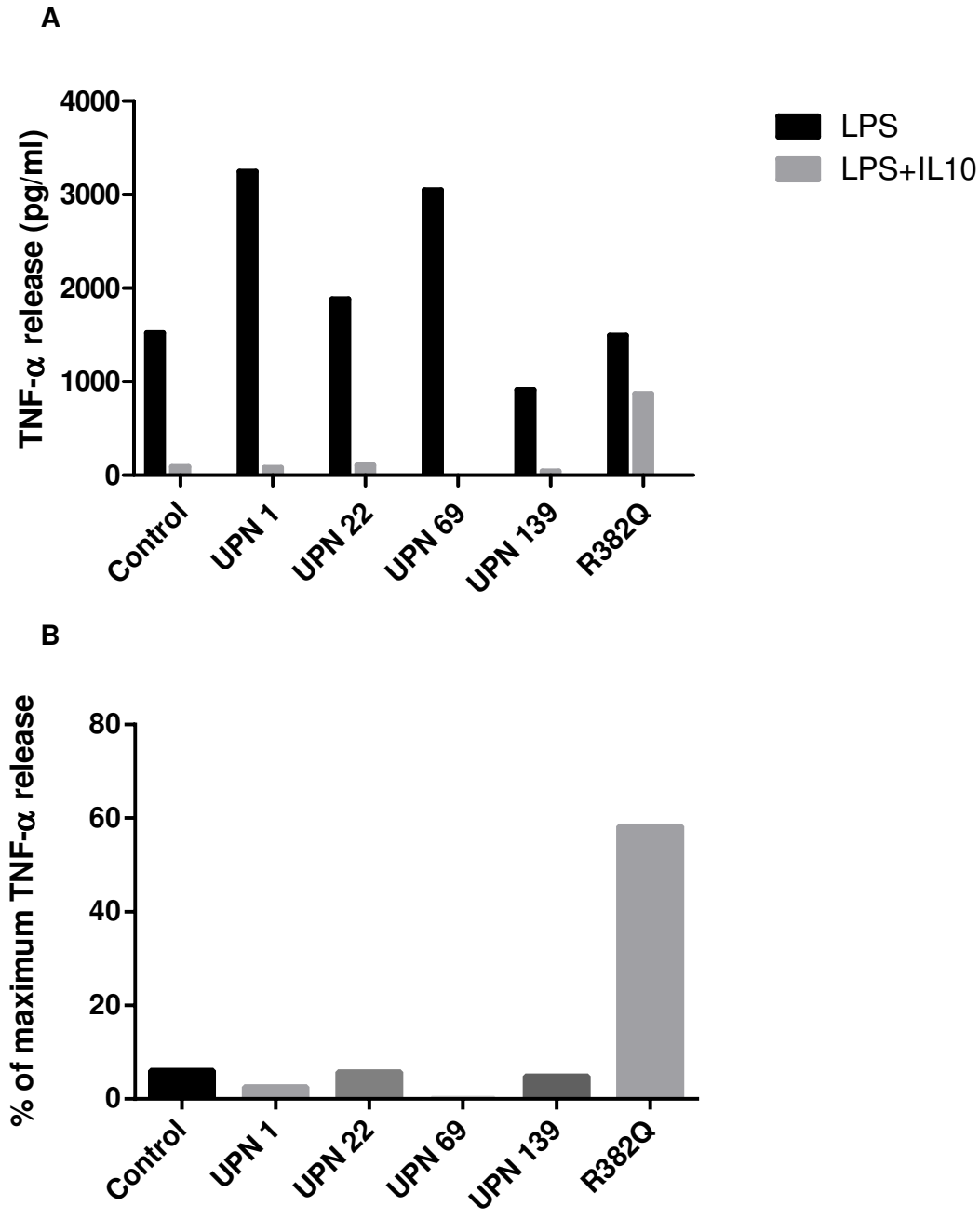


Figure 5.3: Inhibition of TNF-α release in LPS-activated macrophages by IL-10.

Macrophages of four *STAT3*wt patients from Sardinia (UPN1, UPN22, UPN69 and UPN139), one patient with a *STAT3* mutation (R382Q) and two healthy controls were pretreated with IL-10 and then stimulated with LPS. (A) Supernatants were examined for the presence of TNF-α. (B) To ensure better comparability of data from different healthy donors, the impact of IL-10 on TNF-α release is shown as percentage of maximum TNF-α release on LPS stimulation. All four Sardinian patients, as the healthy donor, show almost complete reduction of TNF-α release upon IL-10 treatment indicating unimpaired STAT3 signalling.

5.3.4 Sequencing of *DOCK8*

Even though homozygous mutations in the dedicator of cytokinesis 8 (*DOCK8*) gene have been identified in autosomal-recessive Hyper IgE syndrome (AR-HIES) we sequenced this gene in the Sardinian patients to rule out any so far unknown mutations which may result in an AD-HIES phenotype. Since *DOCK8* is a big gene with 48 exons and three splice variants, the five patients were sequenced on cDNA level with the Ensembl transcript DOCK8-001 ENST00000453981 as reference sequence. None of the patients revealed a mutation, but 12 different single nucleotide polymorphisms (SNPs) were found in exon 2, 3, 6, 11, 16, 20, 24, 32, 36 and 42 either in heterozygous or in homozygous state. All polymorphisms were previously described and are not associated with disease. Information about the variations identified is summarized in Table 5.2. Splice site mutations were ruled out by cDNA sequencing.

5.3.5 Sequencing of components of the IL-6 signalling pathway

To test for other mutations along the IL-6 signalling pathway in the five Sardinian patients, we sequenced the components of the IL-6 receptor (*IL6RA* and *gp130*), *IL-6* itself and *JAK1*.

The *IL-6* gene is located on chromosome 7 and consists of 6 exons, the Ensembl transcript IL6-001 ENST00000404625 was used as reference sequence. No mutation or other sequence variations were detected in any exon of the five patients analyzed. Two heterozygous single nucleotide polymorphisms (SNPs) were found in the intronic regions of three patients. These SNPs have been previously described and are not associated with

disease. Detailed information on all variations detected can be found in the Table 5.2.

The *IL-6RA* gene, the ligand binding part of the IL-6 receptor, consists of 10 exons and is located on chromosome 1. The coding and the adjacent intronic parts of this gene were sequenced on gDNA level and the Ensembl transcript IL6R-001 ENST00000368485 was used as reference sequence. No mutation was detected throughout the whole coding sequence in any of the five patients analyzed. However six variations were found affecting exon 1, 2 and 9 as well as the introns 2 and 4. All variations have been described already and are not disease causing. Each of the five patients carried at least one variation either in a homozygous or a heterozygous state. Detailed information on all variations detected can be found in Table 5.2.

The gene encoding gp130 is located on chromosome 5 and consists of 17 exons. The Ensembl transcript IL6ST-001 ENST00000381298 was used as reference. The coding exons and their adjacent intronic parts were sequenced on gDNA level. No mutation was detected throughout the whole coding sequence in any of the five patients analyzed. Only one sequence variation was found in intron 4 in one patient (see Table 5.2).

JAK1 is located on chromosome 1 and consists of 25 exons. The five patients were sequenced on cDNA level and the JAK1-001 ENST00000342505 Ensembl transcript was used as reference. Patient UPN1 was found to carry a heterozygous mutation in the 5'UTR which has not previously been reported. To confirm the mutation, exon 1 was also sequenced on gDNA in all five patients. The heterozygous change c.-184C>T was detected on genomic DNA in patient UPN1 but in none of the other four patients and none of 100 healthy controls tested (Figure 5.4).

MutationTaster, a free, web-based application for rapid evaluation of the disease-causing potential of DNA sequence alterations (http://www.mutationtaster.org) classified the mutation as disease causing.

The uncle of the patient died in early childhood for unknown reason and the father of the patient is himself prone to illness, therefore all available family members were tested for the presence of the mutation.

The putatively affected father but also two healthy brothers, one sister and the nephew of the patient were all found to carry the same heterozygous variation (Figure 5.4).

Apart from this so far unknown sequence variation found in patient UPN1 nine additional heterozygous SNPs were detected affecting exon 6, 11, 15, 16, 22 and 25. All polymorphisms were previously described and not associated with any disease. Information about the variations found is summarized in Table 5.2. Undetected splice site mutations were excluded by cDNA sequencing.

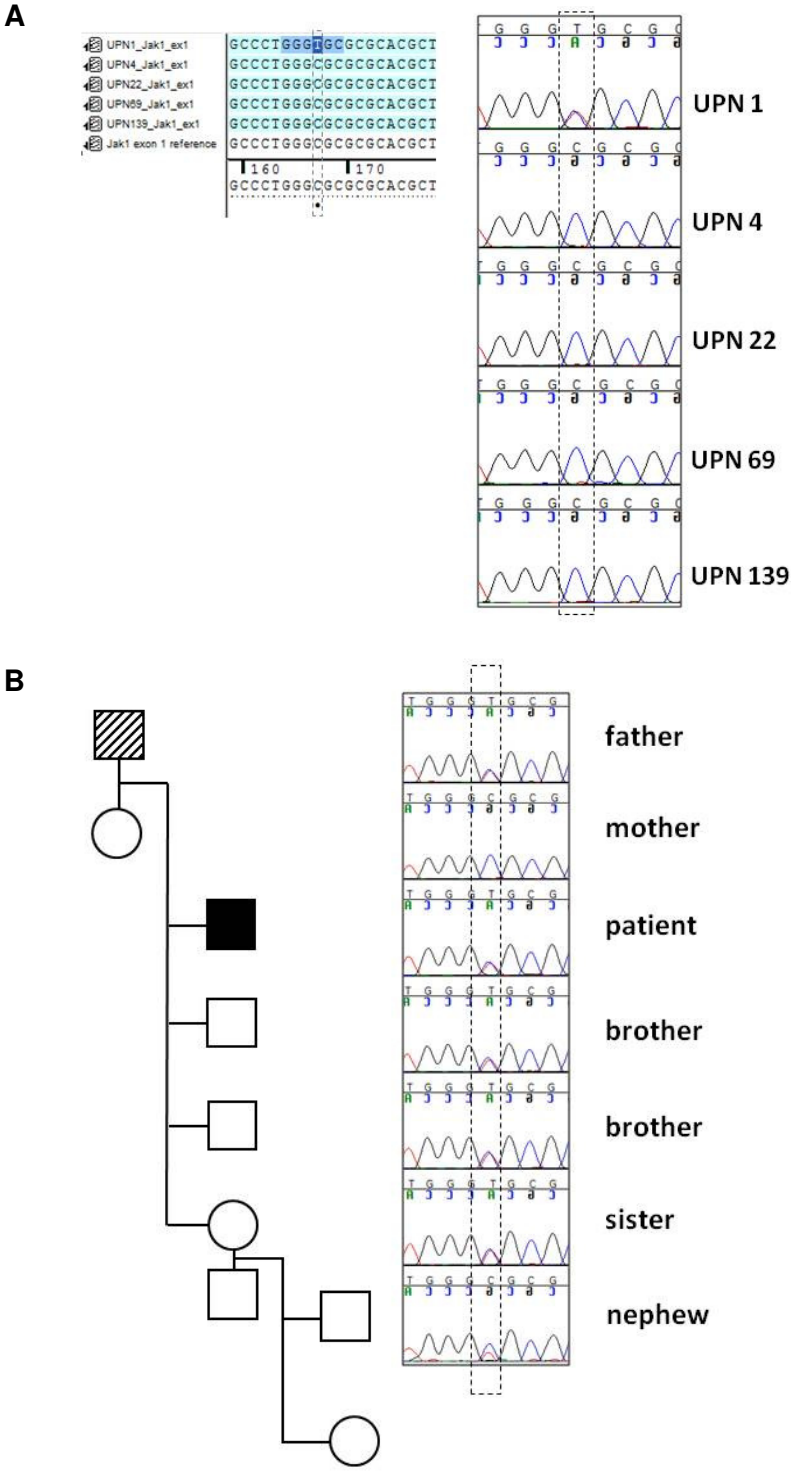


Figure 5.4: *JAK1* sequence variation

(A) Chromatograms of the 5'UTR of *JAK1* of the five Sardinian patients. Patient UPN1 carries a heterozygous sequence change at position c.-184C>T, the other four patients sequenced show wild-type sequence. (B) Pedigree and the relative chromatograms of the family of patient UPN1. The father, both brothers, the sister and the nephew of the patient carry the same sequence variation at position c.-184C>T of *JAK1*. Squares indicate male individuals; circles indicate female individuals. Filled symbols represent patients; the shaded square indicates that the person is possibly affected.

DOCK8					
Name	**Type**	**Location**			**UPN**
rs506121	SNP	exon 2	het	C/T	22
rs3209441	SNP	exon 3	het het	G/A G/A	4 22
rs529208	SNP	exon 3	het het	C/A C/A	1 61
rs2039045	SNP	exon 6	het	T/C	22
TMP_ESP_9_334336	SNP	exon 11	het	A/G	4
rs10970979	SNP	exon 11	hom het het	G A/G A/G	22 61 139
rs913703	SNP	exon 16	het	A/G	22
rs10814431	SNP	exon 20	het	G/C	22
rs2297075	SNP	exon 24	het	C/T	22
rs2297079	SNP	exon 32	hom het	G C/G	4 22
rs7854035	SNP	exon 36	hom hom hom hom	C C C C	4 22 61 139
rs1887957	SNP	exon 42	hom hom het	A A G/A	4 22 139

IL6					
Name	**Type**	**Location**			**UPN**
rs2069827	SNP	47bp before exon 1	het het	G/T G/T	1 22
rs1524107	SNP	intron 3	het	C/T	139

IL6 RA					
Name	**Type**	**Location**			**UPN**
rs4845617	SNP	exon 1	het het het	G/A G/A G/A	1 4 61
rs2228144	SNP	exon 2	het het	G/A G/A	4 139
rs6694817	SNP	intron 2	het het	C/T C/T	4 61
rs7518199	SNP	intron 4	hom hom het hom	C C A/C C	1 22 61 139
rs4845374	SNP	intron 9	het het	T/A T/A	4 139
rs2228145	SNP	exon 9	hom hom het het	C C A/C A/C	1 22 61 139

gp 130					
Name	**Type**	**Location**			**UPN**
rs3729959	SNP	intron 4	het	T/C	139

JAK 1					
Name	**Type**	**Location**			**UPN**
undescribed SNP		exon 1	het	C>T	1
rs45598436	SNP	exon 6	het	T/C	22
rs11585932	SNP	exon 6	het	A/G	139
rs2230586	SNP	exon 11	het	C/T	139
rs3737139	SNP	exon 15	het	C/G	139
rs2230587	SNP	exon 15	het	C/T	61
rs2230588	SNP	exon 16	het	A/G	4
rs12129819	SNP	exon 22	het	G/A	22
rs12353950	SNP	exon 25	het	T/G	22
rs66983374	INSERTION	exon 25	het	-/TTAA	4 22 61

Table 5.2: Sequence variations of the genes sequences.

SNP: single nucleotide polymorphism; het: heterozygous: hom: homozygous; UPN: unique personal number; bp: base pairs

5.3.6 Functionality of mutated JAK1

To examine possible functional consequences of the detected heterozygous mutation c.-184C>T in the 5'UTR of *JAK1* phosphorylation of STAT1 was checked on addition of IFN-α to PBMCs. IFN-α signals via JAK1 and is a strong activator of STAT1.

First cells of healthy controls were stimulated with two different concentrations of IFN-α (100 and 200 ng/mL) for 15 minutes to assess the optimal amount of IFN-α needed for STAT1 phosphorylation. The phosphorylation was detected by immunoblotting with an Anti-phospho STAT1 antibody and was detected with both IFN-α concentrations used (Figure 5.5). About 10^6 PBMCs of two healthy controls, of a *STAT3* mutated (V637M) travel control and of the patient UPN1 were then stimulated for 15 minutes with 100ng/mL IFN-α. STAT1 phosphorylation was detected in all four individuals. STAT1 phosphorylation of the patient, however, appeared to be weaker than phosphorylation in the controls. Since the phosphorylation of the *STAT3* mutated travel control was comparably weak we concluded that this result was most likely due to the transport of the blood samples from Sardinia to London and not caused by an impaired function of JAK1 (Figure 5.5). Hence, we concluded that the mutation in the 5'UTR of *JAK1* of the patient is a rare polymorphism segregating within the family rather than a loss-of-function mutation with functional consequences.

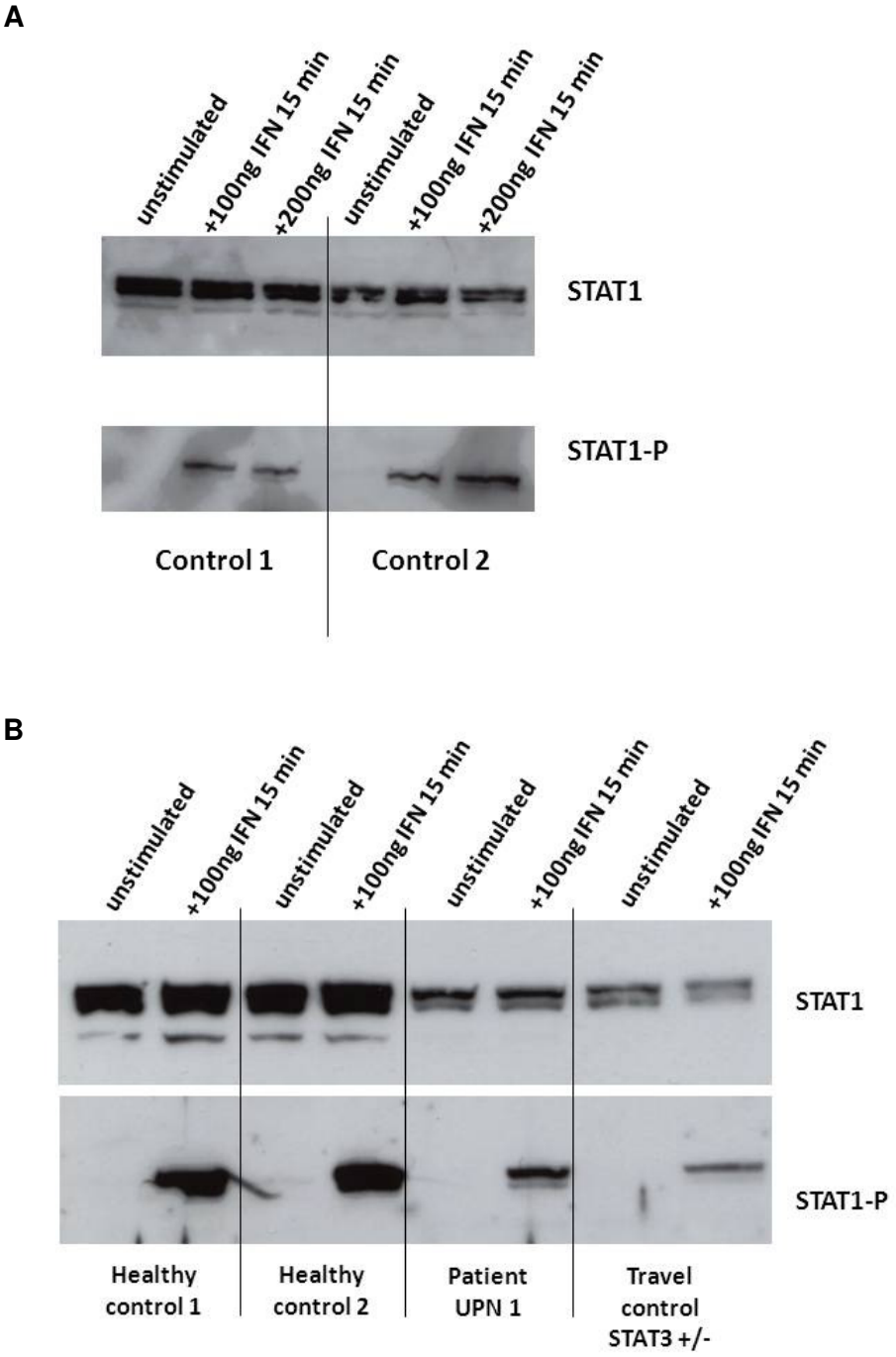


Figure 5.5: STAT1 phosphorylation after IFN-α stimulation

(A) Detection of STAT1 phosphorylation by immunoblotting with an Anti-phospho STAT1 antibody upon stimulation with two different IFN-α concentrations. (B) STAT1 phosphorylation in 10^6 PBMCs of two healthy controls, a *STAT3* mutated patient (travel control) and the patient (UPN1) on stimulation with 100ng/mL IFN-α for 15 minutes.

5.3.7 **Th17 cells**

In the previous chapter we showed that the induction of Th17 cells is impaired in AD-HIES patients with *STAT3* mutations, as it has been described before (de Beaucoudrey et al., 2008; Jiao et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008). We analyzed the levels of Th17 cells among the PBMCs in four of five Sardinian type 1 HIES patients and compared them with 14 control subjects and four *STAT3* mutated patients. Cells were stimulated with PMA/Ionomycin for five hours prior to intracellular staining as described in the methods section. Figure 5.6 shows the stimulation and the gating of one healthy control and one *STAT3* mutated patient. Lymphocytes were first gated for $CD3^+$ and $CD45RO^+$ memory T cells and then on intracellular stained IL-17 and IFN-γ. Unstimulated control cells do neither produce IFN-γ nor IL-17. Stimulated PBMCs of the healthy control showed 33% of IFN-γ-producing and 1.2% of IL-17-producing $CD3^+CD45RO^+$ memory T cells whereas the *STAT3* mutated patient has 21% of IFN-γ-producing but no increase of IL-17-producing $CD3^+CD45RO^+$ T cells. Cells of the four Sardinian patients have inconsistent results (Figure 5.7). UPN22 and UPN69 both had almost 1% of IL-17 positive cells, similarly to the healthy control. UPN1 had intermediate numbers of IL-17 positive cells (0,48%), whereas UPN139 had reduced counts of IL-17 positive cells (0.32%) although they were not completely absent. Numbers of IFN-γ-producing $CD3^+CD45RO^+$ T cells ranged between 16,7 and 21,1% and were therefore in the range of the ones measured for healthy controls. Figure 5.8 summarizes the results of the intracellular staining; the difference of IL-17 positive $CD3^+CD45RO^+$ memory T cell numbers between the healthy controls and the Sardinian patients is not significant ($p=0.1222$).

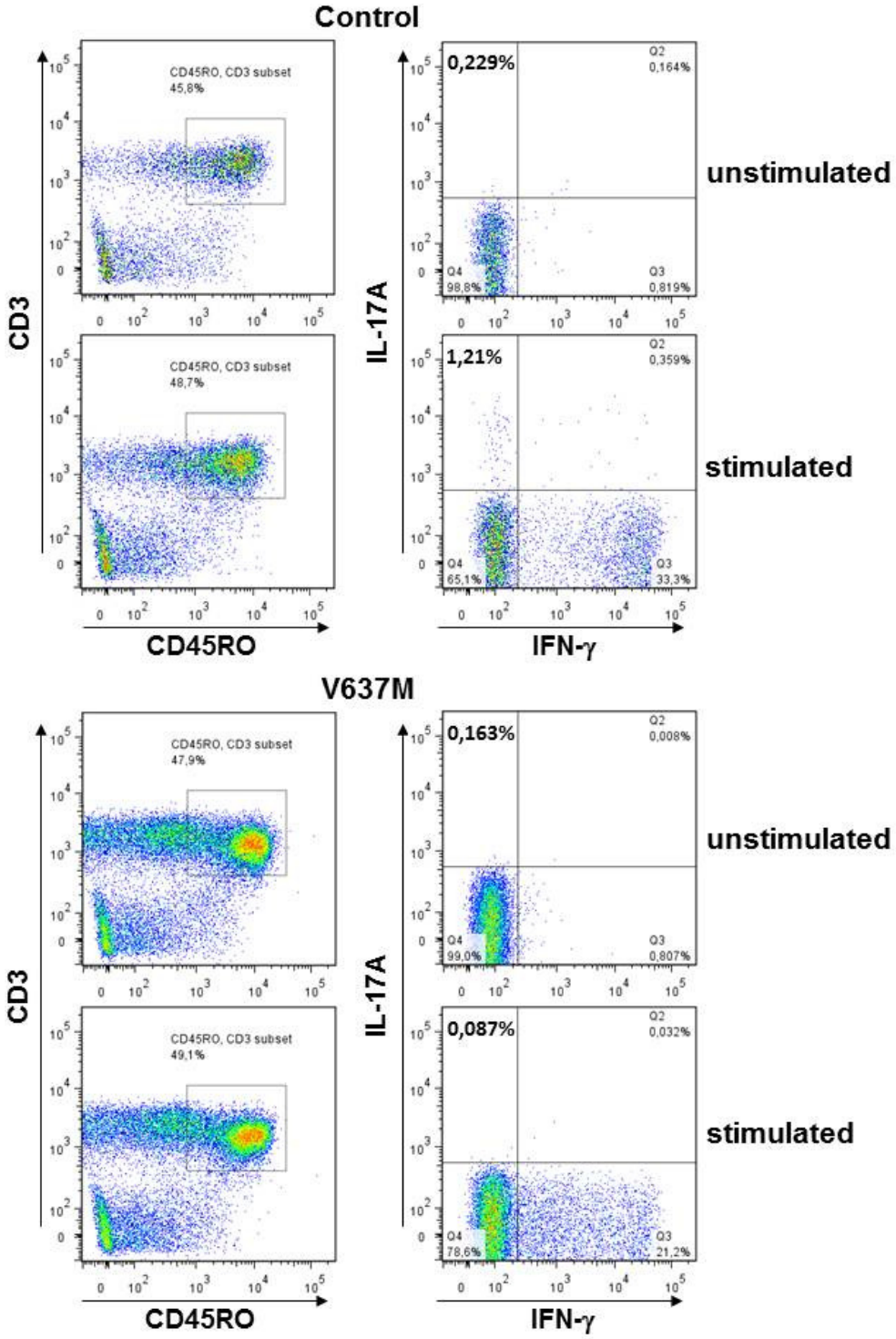


Figure 5.6: IL-17 and IFN-γ production from PBMCs of a healthy control and a STAT3 mutated AD-HIES patient.

Flow cytometry analysis of $CD3^{+}CD45RO^{+}$ memory T cells from a healthy control and *STAT3* mutated AD-HIES PBMCs stained intracellular for IL-17 and IFN-γ. Cells were stained either unstimulated or stimulated with PMA/Ionomycin for 5 hours; Brefeldin A was added after one hour stimulation.

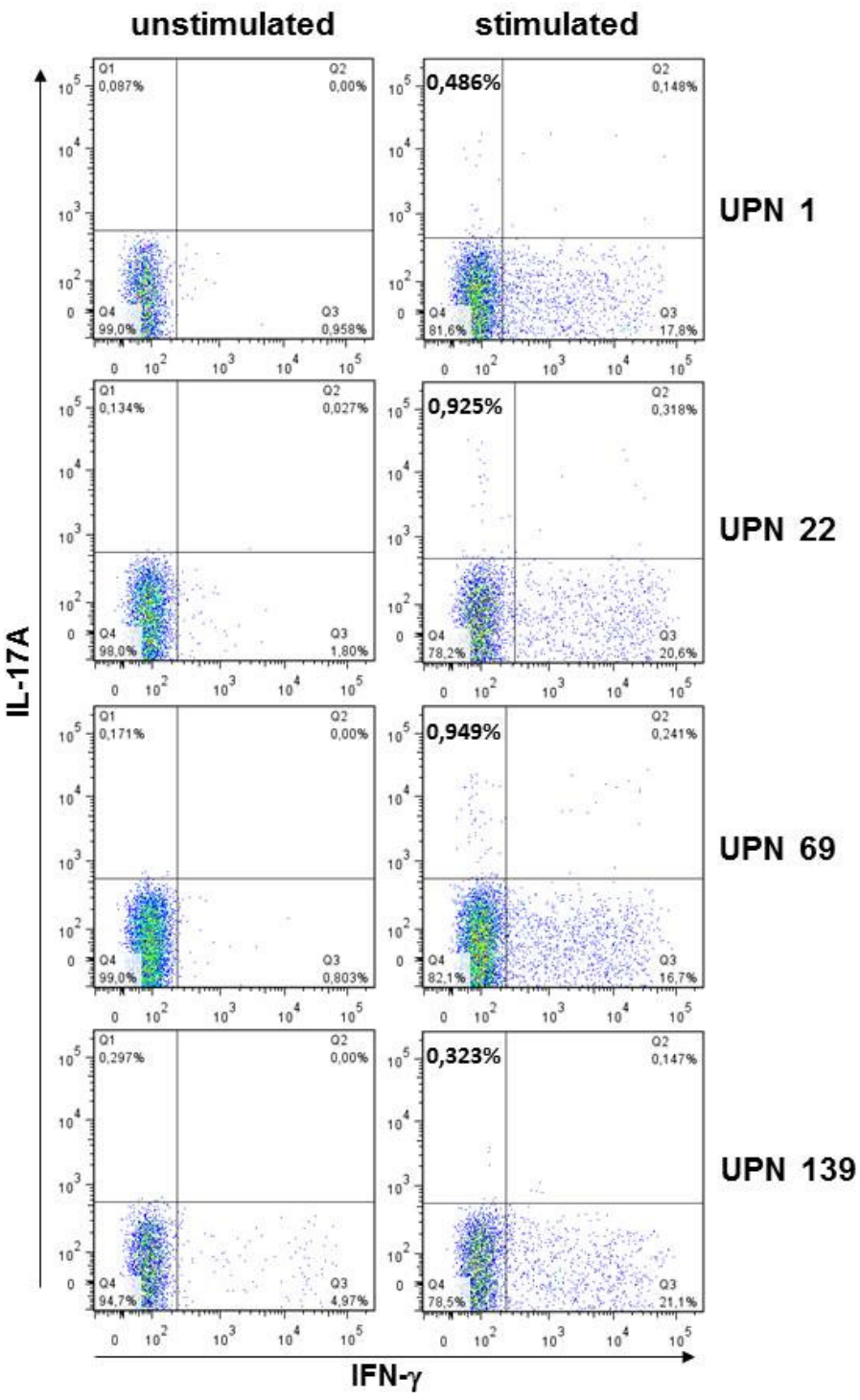


Figure 5.7: IL-17 and IFN-γ production from PBMCs of four Sardinian *STAT3*wt type 1 HIES patients

Flow cytometry analysis of $CD3^+CD45RO^+$ memory T cells from four Sardinian *STAT3*wt AD-HIES patients stained intracellular for IL-17 and IFN-γ. Cells were stained either unstimulated or stimulated with PMA/Ionomycin for 5 hours; Brefeldin A was added after one hour stimulation. Cells of the four Sardinian patients show different results

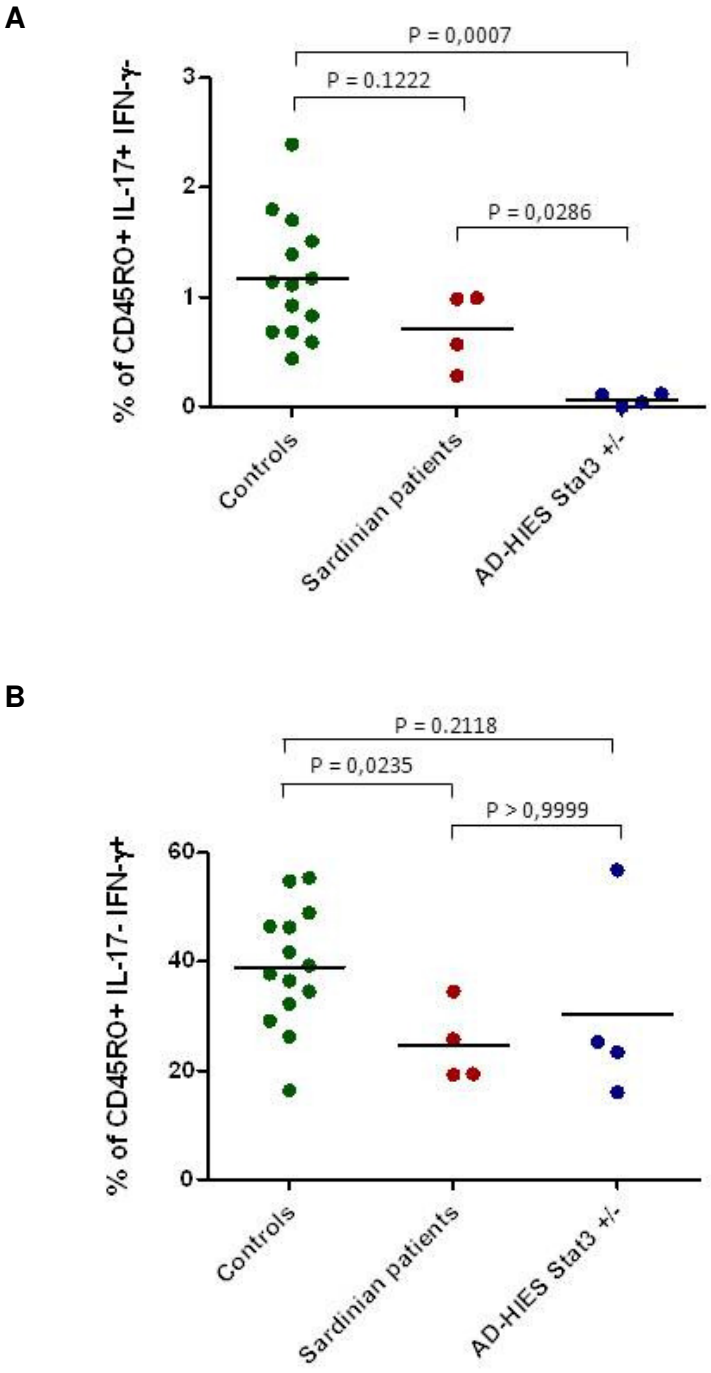


Figure 5.8: Percentage of IL-17 and IFN-γ expressing CD3⁺ T cells

Percentage of (A) IL-17 and (B) IFN-γ expressing $CD3^+CD45RO^+$ memory T cells, determined by intracellular cytokine expression after stimulation with PMA/Ionomycin. Each symbol represents the value from an individual donor or patient. Statistical significance was determined with a Wilcoxon rank-sum test. P values are 2-sided. Median values are shown as horizontal bars.

5.3.8 Multiple cytokine assay

As no mutations with functional consequences were detected in any of the genes sequenced and no clear conclusion could be drawn from the Th17 cell staining, a multiple cytokine assay was carried out to assess whether there is any difference in cytokine production of the Sardinian patients' blood cells when compared to a patient harbouring a known *STAT3* mutation (V637M) and four healthy individuals. For this purpose the FlowCytomix™ Multiple Analyte Detection System (eBioscience, UK) was used. This assay quantifies up to 20 cytokines simultaneously. With the use of this kit the release of 12 different cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-22 and TNF-α) was measured upon stimulation of PBMCs with PMA/Ionomycin for 5 hours.

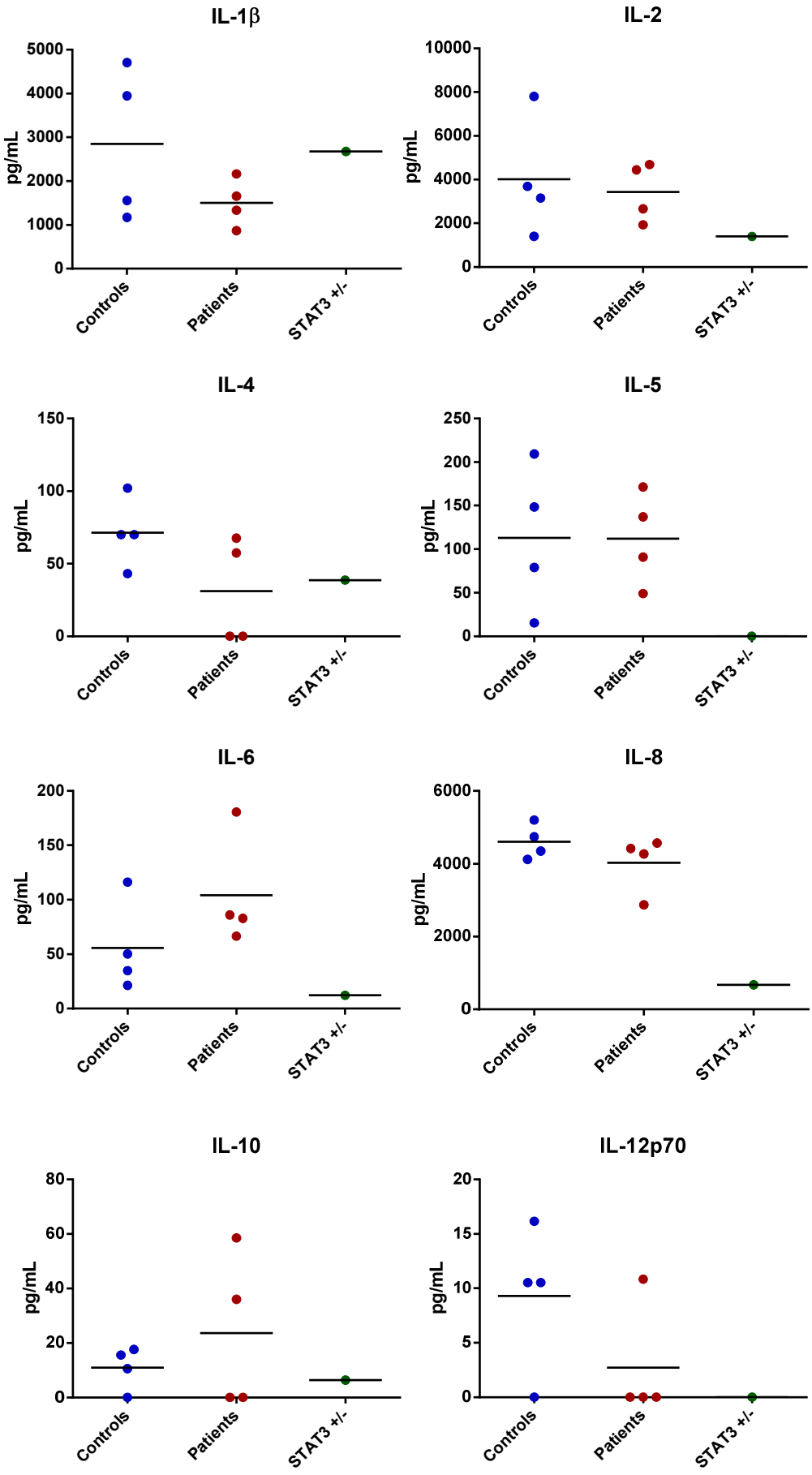
In general no significant difference in cytokine release was detected when comparing the Sardinian patients as a group with the healthy controls. In contrast the *STAT3* mutated patient showed reduced releases of several cytokines (IL-5, IL-6, IL-8, IL-12p70) including, as expected, the production of IL-17 and IL-22 (Figure 5.9).

The Sardinian patients turned out to be a heterogeneous group concerning cytokine release. There was no difference in production of IL-1β, IL-2 and IFN-γ between the healthy controls, the Sardinian patients and the *STAT3* mutated patient. For IL-5 and IL-8 the amount of released cytokines of the Sardinian patients was comparable to the controls, whereas the *STAT3* mutated patient showed a reduced production.

The levels of released IL12p70 and TNF-α were very low and therefore not significant.

Interestingly one Sardinian patient (UPN1) showed higher production of IL-6 when compared with the other Sardinian patients and the controls. Together

with a second patient (UPN69), this patient also had an increased release of IL-10. In contrast the other two patients (UPN22 and UPN139) and one healthy control showed no release of IL-10. In addition to IL-10 the two patients UPN22 and UPN139 show no production of IL-4 and IL-22. The amount of released IL-17 reflects the number of Th17 cells measured by intracellular staining: UPN1, UPN22 and UPN69 showed production of IL-17 comparable to the healthy controls, whereas UPN139, like the *STAT3* mutated patient, lacked IL-17. In general, UPN 139 shows less cytokine release compared to the other Sardinian patients and the healthy controls.


IL-1β
pg/mL
5000
4000
3000
2000
1000
0
Controls
Patients
STAT3 +/-
IL-2
pg/mL
10000
8000
6000
4000
2000
0
Controls
Patients
STAT3 +/-
IL-4
pg/mL
150
100
50
0
Controls
Patients
STAT3 +/-
IL-5
pg/mL
250
200
150
100
50
0
Controls
Patients
STAT3 +/-
IL-6
pg/mL
200
150
100
50
0
Controls
Patients
STAT3 +/-
IL-8
pg/mL
6000
4000
2000
0
Controls
Patients
STAT3 +/-
IL-10
pg/mL
80
60
40
20
0
Controls
Patients
STAT3 +/-
IL-12p70
pg/mL
20
15
10
5
0
Controls
Patients
STAT3 +/-

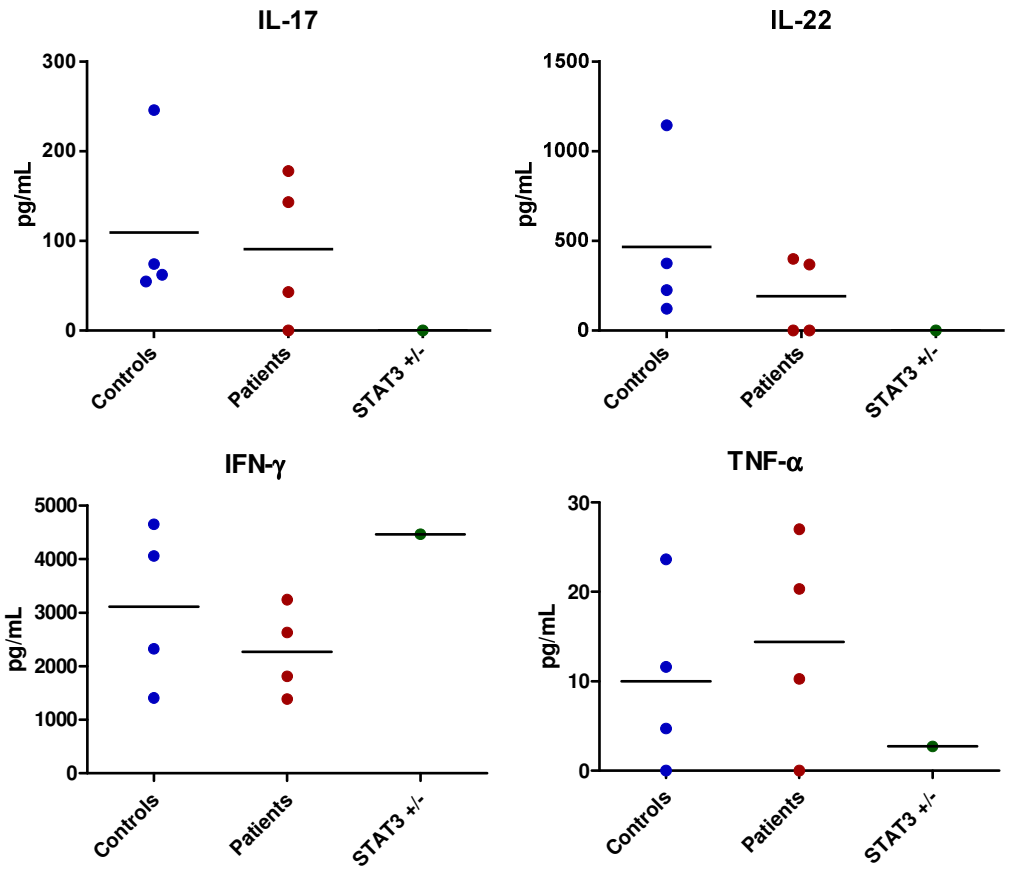


Figure 5.9: Multiple cytokine assay

Amount of cytokines released in the supernatant from PBMCs of four Sardinian *STAT3*wt type 1 HIES patients, four healthy controls and one *STAT3* mutated AD-HIES patient (V637M). Cells were previously stimulated with PMA/Ionomycin for five hours. The levels of the 12 cytokines IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL12p70, IL-17, IL-22, IFN-γ and TNF-α were measured.

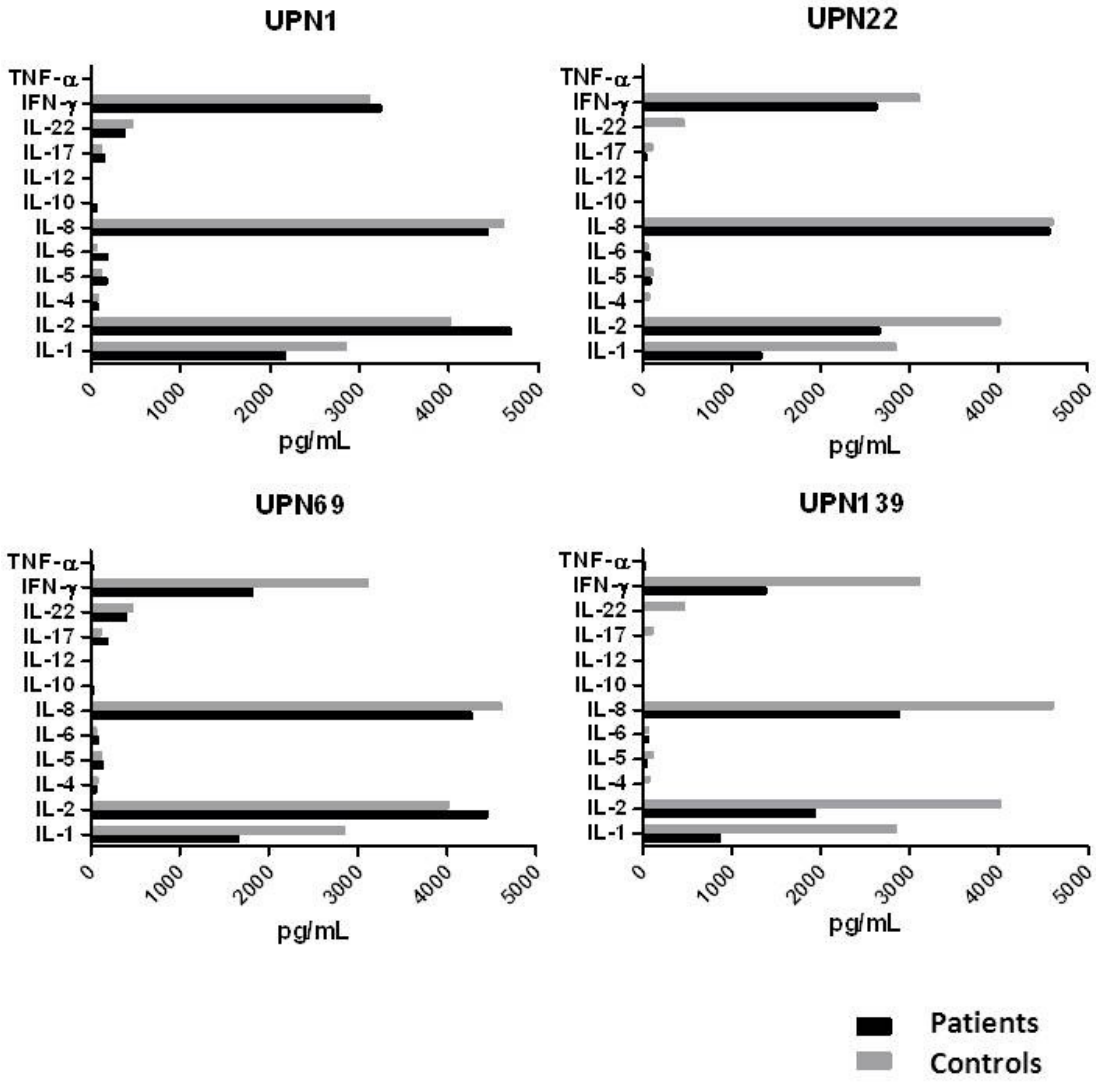


Figure 5.10: Multiple cytokine release

Amount of cytokines released in the supernatant from PBMCs of four Sardinian *STAT3*wt type 1 HIES patients, compared to the mean release values of four healthy controls. Cells were previously stimulated with PMA/Ionomycin for five hours. The levels of the 12 cytokines IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL12p70, IL-17, IL-22, IFN-γ and TNF-α were measured in the supernatant.

5.4 Discussion

A considerable proportion of our patient cohort with the clinical diagnosis of AD-HIES did not show any genetic alteration within the coding exons and the adjacent intronic regions of *STAT3* and *DOCK8*. To shed light on the genetic background of these *STAT3*wt patients with AD-HIES phenotype (type 1 HIES patients), we chose five HIES patients who all originated from Sardinia, one of whom had a putative positive family history of AD-HIES.

Since one case of neutralizing anti-IL-6 autoantibodies has been described in a patient presenting with unusually severe cellulites and subcutaneous abscesses caused by *Staphylococcus aureus* (Puel et al., 2008)for further investigations we focused on components of the IL-6/STAT3 signalling pathway and sequenced *IL-6* itself, the IL-6 receptor components *IL-6RA* and *gp130* and *JAK1*. Despite the assumption that the patient's 'AD-HIES like-phenotype could be due to mutations affecting the IL-6/STAT3 signalling pathway none of the patients revealed a mutation in IL-6, IL-6RA and gp130. One of the patients carried a yet undescribed heterozygous sequence variation in the 5'UTR of *JAK1.* To examine possible functional consequences of the detected mutation phosphorylation of STAT1 was upon addition of IFN-α to PBMCs was investigated, as IFN-α signals via JAK1 and is a strong activator of STAT1. STAT1 phosphorylation of the patient appeared to be weaker than phosphorylation in the controls. However, since the phosphorylation of the *STAT3* mutated travel control was comparably weak we concluded that this result was most likely due to the long travel of the blood samples and not caused by an impaired function of JAK1.

Therefore,this sequence variation most likely is a rare polymorphism with no functional consequence rather than a disease causing mutation. Moreover, healthy family members were found to carry the same variation.

Nevertheless, to assess whether the weaker STAT1 phosphorylation is due to different responsiveness of cells additional controls could have been performed. The simultaneous detection of STAT3 phosphorylation upon addition of IL-6 or IL-10 would have given insight on the general ability of the patient's cells to phosphorylate STAT molecules. Moreover, it would have been advantageous to include a healthy travel control in the analysis.

TYK2 and *JAK2,* which are as well involved in IL-6 signalling, were not sequenced. Even though one of the two TYK2-deficient patients described had elevated IgE levels (Minegishi et al., 2006) the role of TYK2 in the pathogenesis of HIES has been excluded (Woellner et al., 2007). Other authors identified loss-of-function TYK2 mutations in a patient who had an increased susceptibility to infections with Mycobacteria and who did not show any clinical features of HIES (Kilic et al., 2012). Our patients, however, were not prone to mycobacterial infections. Mutations in JAK2 mainly affect the hematopoietic compartment, were shown to cause Polycythemia vera as well as Hereditary Thrombocytosis and myelofibrosis and were therefore excluded *a priory*.

Th17 and IFN-γ-producing $CD3^+CD45RO^+$ memory T cell numbers were investigated in four of the five Sardinian patients. Two patients had normal numbers of Th17 cells as well as IFN-γ-producing memory T cells. One patient had intermediate numbers of these T cell subsets whereas one patient had reduced numbers of Th17. These data correlate with the cytokine release data obtained by the multiple cytokine array. The patient with the reduced number of Th17 cells had also shown reduced IL-17 release after stimulations of his PBMCs. Interestingly, all four patients showed regular STAT3 function at least in response to the immunosuppressive cytokine IL-10 as confirmed by functional tests assessing the IL-10-mediated inhibition of TNF-α release in

LPS stimulated blood cells. Why one patient had reduced Th17 cell numbers remains elusive. Reduced numbers of Th17 cells have been described in other primary immunodeficiencies such as chronic mucocutaneous candidiasis (CMC), a complex disorder characterised by an increased susceptibility to infections with primarily *Candida albicans* (Glocker & Grimbacher, 2011). Reduced Th17 cell numbers in CMC patients can be caused by several mutations hampering the differentiation of Th17 cells from naïve T cells. Loss-of-function mutations in CARD9 result in reduced release of IL-1b, a key cytokine needed for the differentiation towards Th17 cells; gain-of-function mutations in the signal transducer and activator of transcription (STAT)1 enhance cellular responses to cytokines such as IFN-α/β, IFN-γ, and IL-27, which potently inhibit the development of IL-17-producing T cells (Liu et al., 2011).

In general no significant difference in cytokine release was detected when comparing the Sardinian patients as a group with the healthy controls, suggesting that the four patients may not have the same genetic background as assumed. However, two patients together with one control showed no release of IL-10 upon stimulation with PMA/Ionomycin. In addition the same two patients had no release of IL-4 and IL-22. The reason for the diverging cytokine releases remain to be investigated. But, as this experiment has been preformed only once it would beneficial to repeat the experiment possibly applying different stimulation conditions.

In summary, no mutation in select components of the IL-6 signalling pathways that might account for the phenotype of the five patients chosen was found. Even though the Sardinian patients turned out to be a heterogeneous group and showed different cytokine release patterns, they all suffered from candidiasis indicating an impairment of the antifungal defence. Only one patient, however, revealed reduced numbers of Th17 cells which have been

shown to be critical to fight fungal infections. This clearly shows that other immunomodulatory signalling pathways might be impaired in these patients and further studies are needed to examine these pathways.

Recently, dominant gain-of-function mutations in *STAT1* were shown to cause chronic mucocutaneous candidiasis (CMC). These mutations result in impaired STAT1 dephosphorylation and diminished numbers of Th17 cells (Liu et al., 2011b). As CMC and reduced numbers of Th17 cells are two hallmark of the AD-HIES phenotype, it would be worth testing wild type *STAT3* patients for the presence of such gain-of function *STAT1* mutations.

The development of new sequencing technologies such as next-generation sequencing techniques may benefit the search for other type 1 HIES causing genes. These novel methods allow for the rapid examination of multiple genes at considerably lower costs compared to conventional Sanger sequencing techniques. In the near future, candidate gene sequencing might be replaced by whole exome sequencing which enables the simultaneous analysis of exons and/or coding regions only of thousands of genes. This method is not only quicker but it also helps save resources, is more cost efficient and may accelerate the future search for disease causing mutations in HIES or other diseases.

Chapter 6 : Final discussion

The signal transducer and activator of transcription 3 (STAT3) is part of the JAK-STAT signalling pathway, mediating the expression of a variety of genes in response to stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis. STAT3 is known to transduce signals of the entire IL-6-family of cytokines (IL-6, IL-11, IL-27, IL-31), the Leukemia inhibitory factor (LIF), Oncostatin M (OSM), the Ciliary neurotrophic factor (CNTF) and cardiotrophin-1, the IFN-family of cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IFN-α/β and IFN-γ), the IL-2-family of cytokines (IL-2, IL-7, IL-9, IL-15 and IL-21), IL-5, IL-23, the Granulocyte colony-stimulating factor (G-CSF), the Epidermal growth factor EGF, the Macrophage colony-stimulating factor (M-CSF) and Leptin (Darnell, 1997; Levy and Lee, 2002).

In 2007 it has been shown by two different groups that mutations of STAT3 lead to the autosomal dominant form of Hyper IgE Syndrome (AD-HIES) (Minegishi et al., 2007; Holland et al., 2007)

AD-HIES is a rare primary immunodeficiency characterized by both immunologic and non-immunologic manifestations. High serum IgE levels, eosinophilia, eczema, recurrent severe skin and lung infections constitute the immunologic profile of AD-HIES, whereas characteristic facial appearance, scoliosis, retained primary teeth, joint hyperextensibility, recurrent bone fractures following minimal trauma, and craniosynostosis are the main non-immunologic manifestations (Grimbacher et al., 1999a).

The first *STAT3* mutations described, affected the DNA-binding or the SH2 domain of the protein and it soon became apparent that most mutations cluster together and form mutational “hot spots”. All variations found were heterozygous missense mutations or in-frame deletions or duplications, associated with the expression of the protein and exerting a dominant-negative effect (Minegishi et al., 2007).

We sequenced 153 patients with suspected AD-HIES, referred to us in a worldwide collaboration, and found 39 different *STAT3* mutations in 82 patients. Fifty-five patients carried mutations described previously, (de Beaucoudrey et al., 2008; Holland et al., 2007; Jiao et al., 2008; Ma et al., 2008; Milner et al., 2008; Minegishi et al., 2007; Renner et al., 2008) 27 patients harboured novel mutations.

Most mutations affected either the DNA-binding or the SH2 domain, but we also detected one mutation in the N-terminal domain and seven in the transcription activation domain.

All mutations were heterozygous missense mutations or small in-frame deletions or duplications.

The fact that all STAT3 mutations identified in patients with AD-HIES to date are associated with protein expression supports the hypothesis that co-expression of wild-type and mutant STAT3 protein is required to cause AD-HIES. The notable lack of nonsense or frame shift mutations strengthens the notion that AD-HIES is the result of a dominant-negative effect as suggested by Minegishi et al. 2007.

The evidence that the STAT3 mutations are located in areas of the protein known to exert distinct functions suggests that more than one molecular mechanism leads to the same clinical presentation. It has been shown that mutations in the DNA-binding domain cause a defective binding of STAT3 to the DNA (Minegishi et al., 2007) whereas mutations affecting the SH2 domain impair the activation-induced tyrosine phosphorylation of STAT3. The molecular mechanism by which heterozygous mutations in the N-terminal domain or the transcription activation domain of *STAT3* cause the AD-HIES phenotype remains unclear.

The N-terminal domain is conserved among the STATs and is known to be required for the formation of unphosphorylated STAT3 dimers (Vogt et al.,

2011). The lack of nuclear accumulation of STAT3 upon phosphorylation seen in STAT3 variants missing the N-terminal domain could be the underlying mechanism of mutations in this domain leading to the AD-HIES phenotype. The transcriptional activation domains, on the other hand, are regions of a transcription factor which in conjunction with the DNA binding domain can activate transcription by getting in contact with the transcriptional machinery, either directly or through other proteins known as co-activators (Darnell, 1997). How mutations in this region affect the functionality of STAT3 is not understood and further studies will be required to elucidate the mechanism.

Although the molecular mechanism of the impaired STAT3 function appears to be different depending on the domain bearing the mutation, like others, we did not observe any significant correlation between the site of mutation and the patients phenotype (Heimall et al., 2011; Renner et al., 2008) (this analysis was performed in collaboration with the National Centre for Biotechnology Information at the National Institutes of Health (NIH) in Bethesda (USA)) However, the low number of patients carrying mutations outside the DNA-binding or the SH2 domain makes statistical analysis difficult. Thus, phenotypic differences may not become apparent until more patients with AD-HIES with these STAT3 mutations have been characterized.

Since the mutations found in STAT3 can be spread over the entire gene, it is necessary to sequence the whole *STAT3* gene to exclude possible mutations. It therefore would be desirable to find a test capable of predicting a mutation without the need of sequencing.

For some of the mutations we found an impairment of the IL-10-mediated inhibition of TNF-α release in LPS challenged patients' macrophages, indicating a failure in the IL-10/Janus kinase/STAT3 pathway. This effect,

however, was less prominent in some of the mutations tested, in particular in the four novel mutations: H58Y in the N-terminal domain; N466K in the DNA-binding domain; and P639S and F710C affecting the SH2 domain. Furthermore, we experienced great variability in TNF-α release for some *STAT3* mutations; this makes this assay unsuitable as screening method to identify patients with *STAT3* mutations. Additionally, one should bear in mind that the *STAT3* mutations leading to the AD-HIES phenotype are heterozygous, heterogeneous and display a variable functional deficit. Therefore, it is difficult to set up a specific test capable of predicting all mutations.

As mutations in STAT3 lead to impaired Th17 cell differentiation, significantly reduced numbers of Th17 cells are a common feature of AD-HIES patients carrying STAT3 mutations (de Beaucoudrey et al., 2008; Jiao et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008). Th17 cells are positioned at the intersection between innate and adaptive immune response and play a significant role in the defence against extracellular and intracellular pathogens (Peck and Mellins, 2010).

Similar to previous reports we found that our AD-HIES patients carrying a STAT3 mutation had markedly decreased or absent numbers of circulating Th17 cells, suggesting a fundamental defect in Th17 cell differentiation.

Reduced numbers of Th17 cells are thought to contribute most to the clinical phenotype of AD-HIES including to the increased susceptibility to bacterial and fungal infections. The relevance of Th17 cells in antibacterial and antifungal host defence has been demonstrated in other human diseases and significantly reduced numbers of Th17 cells are implicated in the pathogenesis of Chronic mucocutaneous candidiasis (CMC) (Glocker and Grimbacher, 2011). Th17 cells also secrete IL-22, a cytokine that upregulates the secretion

of antimicrobial peptides like human β-defensins and CCL20 (Kolls et al., 2008) which supports the critical role of Th17 cells for protecting skin and mucosal surfaces.

In contrast to their role in protective immunity against fungi, Th17 cells have been shown to be involved in auto-immune disease, by exerting uncontrolled excessive tissue damage.

Enumeration of IL-17 production by activated CD4 T cells emerges as a relatively quick and simple procedure that could be used to screen patients suspected of having AD-HIES due to inactivating mutations in *STAT3*.

Moreover, future studies of individuals with AD-HIES may also help to explain the role of Th17 cells in infection and in other forms of dysregulated immunity, and may help in understanding the role of STAT3 in T-helper cell differentiation in humans. Furthermore, our findings suggest that therapeutic approaches to AD-HIES may focus on therapies improving and increasing the IL-17 production.

Reports that haematopoietic stem cell transplantation (HSCT) may correct this deficit underline the relevance of this T cell subset in the pathogenesis of HIES (Goussetis et al., 2010).

One of the *STAT3* mutated AD-HIES patients of our cohort underwent haematopoietic stem cell transplantation in 2005 at the age of 13. The patient carried the 'common' mutation R382W and despite prophylactic antibiotic treatment he suffered from recurrent chest infections with longstanding established bronchiectasis. The expectation of the transplant was to at least cure the immunological features of the syndrome by replacing the haematopoietic system.

To date three haematopoietic stem cell transplantation attempts have been reported in literature (Gennery et al., 2000; Goussetis et al., 2010; Nester et al., 1998).

The first report concerned an adult AD-HIES patient who developed lymphoma. The patient died of transplantation-related complications 6 months after the procedure but showed decreased serum IgE levels and fewer HIES-related symptoms (Nester et al., 1998).

The authors of the second report described a 7 year-old girl who experienced a relapse of AD-HIES 4 years after transplantation, despite full donor chimerism in all leukocyte lineages (Gennery et al., 2000). To date the patient is still alive and well (personal communication of the authors A. Gennery and A. Cant).

The latest report depicts the cases of two unrelated boys with AD-HIES complicated as well by high-grade non-Hodgkin lymphoma. Already two month after transplantation the authors measured normal IgE which remained normal 14 and 10 years after transplantation for both patients respectively. Additionally, the authors state that neither patient had any additional pneumonia, skin infection, or other problem associated with AD-HIES following the transplant. AD-HIES related nonimmunologic manifestations, including skin lesions and osteoporosis also resolved and the coarse appearance of the skin of the face with pronounced pores gradually disappeared. Th17 cells levels were measured in both patients. IL-17-producing cell populations were found to be 0.8% of CD4 cells, and therefore comparable to healthy donors (Goussetis et al., 2010).

In our patient we showed as well that the number of circulating Th17 cells was restored after transplantation, indicating the importance of Th17 cells in the pathogenesis of AD-HIES with STAT3 mutations. Indeed the patient has significantly improved, he suffered from less infections and the progressive

lung destruction has been stalled. Therefore, we conclude that allogeneic HSCT represents a curative treatment for patients with AD-HIES presenting with progressive lung disease leading to the prevention and even reversal of AD-HIES manifestations.

A considerable proportion of our patient cohort (71 out of 153) with the clinical diagnosis of AD-HIES did not show any genetic alteration within the coding exons and the adjacent intronic regions of STAT3. Mutations in the promoter region were ruled out in advance: even though conceivable they were unlikely to cause AD-HIES when present in heterozygosity because they would not exert a dominant-negative effect which seems to be required for the AD-HIES phenotype.

As no inheritance pattern has been identified for patients, resembling an autosomal dominant HIES phenotype but without proven STAT3 mutation we decided to refer to these STAT3wt patients as type 1 HIES instead of AD-HIES**.**

To shed additional light on the genetic background of STAT3wt type 1 HIES we selected five patients who all originated from Sardinia.

We focused on components of the IL-6/STAT3 signalling pathway and sequenced IL-6 itself, the IL-6 receptor components IL-6RA and gp130 and JAK1. None of the patients harboured a mutation in IL-6, IL-6RA and gp130, but one of them carried a yet undescribed heterozygous variation in the 5'UTR of JAK1. Functional assays, however, showed that this variation most likely is a rare polymorphism rather than a disease causing mutation with functional consequences. Moreover, healthy family members were found to carry the same variation.

The numbers of Th17- and IFN-γ-producing memory T cells were examined in four of these five Sardinian patients. Two patients had normal numbers of

these T cell subsets, one patient had slightly decreased and one patient had clearly reduced cell numbers. These data correlated with the results of the multiple cytokine arrays. The patient with the reduced Th17 cell numbers also showed reduced IL-17 release upon stimulation of his PBMCs with PMA and Ionomycin. Interestingly, all four patients showed regular STAT3 function as confirmed by the capability of IL-10 to inhibit the TNF-α release in LPS stimulated leucocytes. The reason why one patient had reduced Th17 cell numbers remains to be elusive. These results confirm the heterogeneity of HIES and show that signalling pathways other than the IL-6/STAT3 pathway might be affected. Thus further studies are needed to examine the genetic background of type 1 HIES with wild-type STAT3 by using other strategies such as SNP chip analyses, whole exome sequencing, gene expression arrays and ELISAs reading out various pro- and anti-inflammatory cytokines.

Heterozygous missense gain-of-function mutations of *STAT1* were recently identified in patients with chronic *Candida* infections, autoimmune manifestations and mild bacterial or viral diseases (Liu et al., 2011a). In these patients, the development of Th17-cells is impaired, probably due to enhanced STAT1-dependent cellular responses to repressors of Th17 cell differentiation (Puel et al., 2012). As CMC and reduced numbers of Th17 cells are two hallmarks of the HIES phenotype, it would be worth testing wild type *STAT3* patients for the presence of such gain-of function *STAT1* mutations.

During the course of this study it also became clear that homozygous mutations of the dedicator of cytokinesis 8 (DOCK8) gene are responsible for the autosomal recessive form of HIES (AR-HIES) (Engelhardt et al., 2009; Zhang et al., 2009). Four patients out of our cohort of 71 *STAT3*wt HIES patients erroneously classified as autosomal dominant were found to carry

DOCK8 mutations suggesting the importance and value of the complete medical and family history.

The work presented in this thesis has characterized patients with AD-HIES, the frequency of STAT3 mutations in AD-HIES and functional consequences of STAT3 mutations. Thus, the data presented contribute to a better understanding of the AD-HIES as well as the STAT3 signalling and its importance in immunity and T cell development. The results obtained may pave the way for alternative future therapeutical approaches such as HSCT or gene therapy -based treatment of AD-HIES, thereby improving the clinical management of affected patients.

Many fundamental aspects of STAT3 biology are still unanswered but the wide repertoire of mutations identified within the STAT3 gene represents a suitable tool to perform structure/function studies on STAT3 and gives the opportunity to investigate fundamental aspects of STAT3 biology.

Although the AD-HIES phenotype can partially be explained, the exact molecular mechanism by which heterozygous mutations in STAT3 cause AD-HIES remains unclear.

The paradoxical pro- and anti-inflammatory functions of STAT3 are still challenging questions that are linked to the understanding of the immunological and non-immunological features of AD-HIES. It will be interesting to explore the exact underlying molecular mechanisms of specific mutations and how these affect the function of STAT3. Moreover, the role of unphosphorylated STAT3 molecules in the pathogenesis of AD-HIES as well as the impact of STAT3 mutations on other JAK-STAT signalling pathways and their role in the formation of STAT1-STAT3 heterodimers requires further investigation.

Theoretically, mutations altering the function of any receptors, signalling pathways, and cytokines related to STAT3 may cause diseases that are more or less similar to classical AD-HIES. These pathways should therefore be further analyzed in type 1 HIES patients without *STAT3* mutations.

In general, the proposed work generated basic knowledge of the molecular biology of STAT3 signalling in AD-HIES patients and thus may have paved the way to develop alternative therapies for patients suffering from either AD-HIES or other diseases presenting with imbalanced Th17 cells.

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Appendix

UPN	Gender	Age of scoring	Origin	Mutation	Domain	NIH score*	recurrent skin abscesses	recurrent pneumonia	lung cyst formation	other unusual infections	eosinophilia	newborn rash	eczema	recurrent upper respiratory tract infections	candidiasis	failure to shed deciduous teeth	scoliosis	pathologic bone fractures	hyperextensibility	characteristic face for Job syndrome	increased inter-alar distance	cathedral palate	midline anomaly
94**	**F**	11	EU	H58Y	N-terminal	42	yes	no	no	no	<700	yes	severe	3	no	>3	<10°	no	yes	no	no	no	no
7	**F**	12	EU	a→g ex 12 (-2)	DNA-B	53	yes	yes	yes	no	>800	no	moderate	1-2	nail	2	<10°	no	yes	yes	nd	nd	no
30	**F**	14	SA	a→g ex 12 (-2)	DNA-B	48	yes	yes	yes	yes	>800	yes	moderate	4-6	oral	nd	<10°	no	no	no	yes	no	no
114	**M**	22	EU	a→g ex 12 (-2)	DNA-B	70	yes	yes	yes	yes	>800	yes	severe	4-6	oral	nd	10-14°	nd	nd	yes	yes	yes	yes
86	**M**	13	EU	g→a ex 12 (-1)	DNA-B	83	yes	yes	yes	yes	<700	yes	severe	4-6	oral	>3	10-14°	yes	yes	yes	nd	yes	yes
17	**F**	25	EU	g→t ex 12 (+1)	DNA-B	65	yes	yes	yes	yes	700-800	nd	severe	1-2	nail	>3	nd	no	no	yes	yes	yes	no
160**	**F**	10	EU	g→a ex 12(+1)	DNA-B	40	yes	no	yes	yes	>800	yes	moderate	4-6	no	0	<10°	no	no	no	no	no	no
108	**M**	14	EU	ins t ex 12 (+2)	DNA-B	54	no	yes	yes	yes	>800	no	moderate	1-2	systemic	>3	<10°	no	no	mild	no	yes	no
70	**F**	34	EU	982 CMP ins	DNA-B	41	yes	yes	no	no	<700	no	moderate	3	oral	2	10-14°	no	yes	yes	no	yes	no
110**	**F**	28	EU	G342D	DNA-B	79	yes	yes	yes	yes	700-800	yes	severe	4-6	nail	>3	nd	yes	yes	yes	yes	yes	no
79	**F**	8	EU	R382L	DNA-B	56	yes	yes	no	no	>800	yes	severe	3	nail	3	<10°	yes	nd	yes	nd	nd	no
98	**M**	25	EU	R382L	DNA-B	45	yes	yes	yes	yes	>800	no	moderate	3	oral	nd	<10°	no	no	yes	nd	no	no
25	**F**	11	EU	R382Q	DNA-B	72	yes	yes	yes	yes	<700	yes	severe	>6	systemic	>3	<10°	yes	yes	mild	no	no	no
37	**F**	6	EU	R382Q	DNA-B	46	no	yes	no	no	<700	nd	severe	>6	no	>3	<10°	no	yes	yes	yes	yes	no
44	**M**	9	ME	R382Q	DNA-B	62	yes	yes	yes	no	700-800	yes	moderate	>6	nd	>3	<10°	no	yes	mild	yes	no	no

47	**F**	24	ME	R382Q	DNA-B	62	yes	yes	yes	yes	>800	yes	severe	1-2	no	nd	nd	yes	no	yes	yes	no	nd
61	**M**	14	ME	R382Q	DNA-B	57	yes	yes	yes	nd	nd	no	severe	>6	nail	>3	nd	no	no	yes	nd	yes	no
72	**M**	19	ME	R382Q	DNA-B	60	yes	yes	yes	nd	700-800	yes	severe	4-6	systemic	>3	nd	nd	nd	no	yes	nd	nd
77	**M**	19	ME	R382Q	DNA-B	61	yes	yes	yes	yes	700-800	nd	moderate	>6	nail	0	nd	yes	nd	yes	yes	nd	no
92	**M**	12	ME	R382Q	DNA-B	50	yes	yes	yes	nd	<700	yes	severe	nd	nd	nd	nd	nd	yes	yes	yes	no	nd
95	**F**	15	EU	R382Q	DNA-B	48	yes	yes	yes	yes	700-800	nd	moderate	1-2	nd	nd	nd	yes	yes	mild	yes	nd	nd
164	M		EU	R382Q	DNA-B		no detailed clinical information available																
183	M		EU	R382Q	DNA-B		no detailed clinical information available																
15	**M**	29	EU	R382W	DNA-B	76	yes	yes	yes	yes	>800	yes	severe	1-2	nail	>3	nd	yes	nd	yes	yes	yes	no
19**	**M**	15	EU	R382W	DNA-B	63	yes	yes	yes	yes	<700	yes	severe	1-2	nail	>3	<10°	yes	yes	mild	yes	yes	no
29	**F**	29	SA	R382W	DNA-B	64	yes	yes	yes	yes	>800	yes	moderate	4-6	nail	>3	<10°	no	no	yes	yes	no	no
31	**M**	12	SA	R382W	DNA-B	57	yes	yes	yes	yes	>800	yes	moderate	4-6	nail	3	nd	yes	no	no	yes	no	no
43	**M**	20	ME	R382W	DNA-B	62	yes	yes	yes	no	nd	yes	severe	4-6	oral	>3	<10°	no	yes	mild	yes	yes	no
56	**M**	15	EU	R382W	DNA-B	59	yes	yes	yes	no	>800	yes	severe	nd	no	>3	<10°	yes	no	yes	nd	nd	no
63	**M**	28	ME	R382W	DNA-B	61	yes	yes	yes	no	>800	yes	mild	1-2	nail	>3	<10°	no	yes	mild	no	no	no
73	**M**	17	ME	R382W	DNA-B	73	yes	yes	yes	yes	700-800	yes	severe	nd	nd	>3	15-20°	nd	yes	yes	yes	nd	nd
126	**F**	11	EU	R382W	DNA-B	58	yes	yes	yes	yes	>800	yes	mild	3	oral	0	10-14°	no	no	mild	yes	yes	nd
128	**F**	3	EU	R382W	DNA-B	29	yes	yes	no	no	<700	no	moderate	1-2	nail	0	<10°	yes	no	mild	yes	no	no
129	**M**	11	EU	R382W	DNA-B	64	yes	yes	yes	yes	<700	no	severe	1-2	systemic	>3	<10°	yes	no	yes	yes	yes	no
135	**M**	7	EU	R382W	DNA-B	60	yes	yes	yes	yes	700-800	yes	severe	3	oral	nd	nd	no	yes	yes	nd	yes	no
136	**F**	15	ME	R382W	DNA-B	62	yes	yes	yes	nd	>800	yes	severe	>6	nail	0	<10°	no	nd	yes	yes	no	no
162	M		EU	R382W	DNA-B		no detailed clinical information available																
185	F		EU	R382W	DNA-B		no detailed clinical information available																
6	**F**	50	EU	F384L	DNA-B	54	yes	yes	yes	no	<700	yes	severe	nd	no	0	10-14°	yes	no	yes	yes	no	no
60**	**M**	29	ME	F384L	DNA-B	71	yes	yes	yes	yes	>800	nd	mild	>6	nail	>3	<10°	yes	yes	yes	yes	yes	nd
154	**F**	3	EU	F384L	DNA-B	20	no	no	no	no	nd	yes	moderate	1-2	no	3	<10°	no	no	no	no	no	no
65**	**M**	23	ME	T389I	DNA-B	52	yes	yes	no	no	>800	yes	severe	1-2	nail	2	<10°	no	no	yes	yes	no	no
143	**F**	16	EU	T412A	DNA-B	69	yes	yes	yes	yes	>800	no	moderate	1-2	no	nd	>20°	yes	yes	yes	no	yes	no
118	**F**	32	EU	R423Q	DNA-B	59	yes	yes	yes	no	<700	no	severe	4-6	no	0	10-14°	yes	yes	yes	yes	no	yes
130	**F**	14	EU	V463 del	DNA-B	56	no	yes	yes	yes	>800	no	moderate	1-2	no	>3	<10°	no	no	yes	yes	yes	no

26	**F**	11	EU	N466D	DNA-B	55	yes	yes	no	yes	<700	yes	severe	1-2	oral	>3	<10°	no	yes	yes	yes	yes	no
2**	**F**	30	EU	N466S	DNA-B	41	no	yes	yes	no	>800	no	mild	4-6	no	>3	nd	yes	nd	mild	nd	nd	nd
46	**F**	15	ME	N466T	DNA-B	66	yes	yes	yes	no	nd	no	moderate	>6	nd	>3	>20°	no	yes	yes	yes	no	no
102	**M**	12	EU	N466K	DNA-B	45	yes	yes	no	no	<700	no	moderate	>6	oral	>3	<10°	yes	yes	yes	yes	no	no
36	**M**	16	EU	Q469H	DNA-B	57	yes	no	no	yes	nd	yes	severe	1-2	oral	>3	<10°	yes	no	yes	yes	yes	no
57	**F**	18	EU	K591E	SH2	75	yes	yes	yes	yes	>800	yes	severe	1-2	nail	>3	<10°	yes	yes	yes	nd	nd	nd
147	**M**	2	EU	K615E	SH2	32	yes	yes	no	yes	>800	no	moderate	3	nail	nd	nd	no	no	mild	yes	no	no
187	M		EU	K615E	SH2		no detailed clinical information available																
5	**M**	11	EU	T622I	SH2	47	yes	yes	yes	no	>800	no	moderate	1-2	no	2	10-14°	no	no	yes	nd	yes	no
71	**M**	24	EU	S636Y	SH2	71	yes	yes	yes	yes	>800	yes	mild	1-2	oral	2	>20°	no	yes	mild	yes	yes	nd
104	**M**	6	EU	V637A	SH2	72	yes	yes	yes	no	>800	no	severe	>6	nail	>3	<10°	yes	yes	yes	yes	no	no
16	**F**	39	EU	V637M	SH2	68	yes	yes	yes	no	>800	no	severe	4-6	nail	>3	nd	yes	yes	yes	yes	yes	no
32	**M**	15	SA	V637M	SH2	37	yes	yes	no	yes	<700	yes	moderate	3	no	2	<10°	no	no	mild	yes	no	yes
33	**M**	25	SA	V637M	SH2	45	yes	yes	yes	no	<700	no	moderate	4-6	oral	0	<10°	no	no	yes	yes	no	no
42	**F**	11	ME	V637M	SH2	58	yes	yes	yes	yes	>800	no	moderate	1-2	no	>3	nd	no	no	yes	yes	yes	no
45	**M**	9	ME	V637M	SH2	44	yes	yes	yes	no	nd	yes	moderate	4-6	oral	>3	<10°	no	yes	no	yes	no	no
66	**F**	21	EU	V637M	SH2	56	yes	yes	no	yes	<700	no	severe	1-2	nail	>3	<10°	yes	yes	yes	yes	yes	no
67	**M**	12	EU	V637M	SH2	59	yes	yes	yes	yes	>800	yes	severe	1-2	no	1	<10°	yes	nd	mild	no	yes	no
106**	**M**	4	EU	V637M	SH2	30	yes	yes	no	yes	>800	no	moderate	3	no	0	<10°	no	no	mild	yes	no	no
115	**M**	19	EU	V637M	SH2	56	yes	yes	nd	nd	nd	nd	severe	nd	nd	>3	<10°	yes	no	yes	yes	yes	no
125	**M**	16	EU	V637M	SH2	58	yes	yes	yes	yes	>800	yes	severe	4-6	no	nd	10-14°	no	no	yes	yes	no	nd
151	**M**	5	EU	V637M	SH2	47	yes	yes	no	no	>800	yes	severe	1-2	oral	>3	<10°	no	no	no	no	yes	no
186	M		EU	V637M	SH2		no detailed clinical information available																
157	**M**	9	EU	V637L	SH2	42	yes	yes	no	no	<700	yes	mild	1-2	no	0	<10°	yes	yes	yes	nd	no	no
49	**M**	7	NA	P639S	SH2	55	yes	yes	yes	no	<700	yes	severe	4-6	nail	>3	<10°	no	no	mild	no	yes	yes
158	**F**	7	EU	P639R	SH2	46	yes	yes	yes	nd	>800	no	moderate	1-2	oral	>3	<10°	no	yes	mild	nd	yes	no
153**	**M**	43	EU	Y640N	SH2	44	yes	yes	no	yes	<700	no	no	>6	no	>3	<10°	no	no	yes	yes	yes	no
109	**F**	21	EU	Y657C	SH2	33	yes	no	no	yes	>800	no	moderate	3	nail	0	<10°	no	no	no	nd	no	no
175	F		EU	Y657C	SH2		no detailed clinical information available																

27	**M**	15	SA	S668F	SH2	56	yes	yes	yes	yes	>800	yes	moderate	4-6	oral	2	<10°	yes	no	no	yes	no	no
152	**M**	31	EU	Y705H	TAD	79	yes	yes	yes	yes	<700	no	moderate	4-6	nail	>3	15-20°	yes	yes	yes	yes	yes	yes
169	M		EU	L706P	TAD		no detailed clinical information available																	
103	**F**	23	EU	T708S	TAD	53	no	yes	yes	no	>800	yes	severe	1-2	oral	>3	<10°	nd	nd	yes	yes	nd	no
159	**F**	18	EU	T708S	TAD	54	yes	yes	yes	nd	nd	nd	moderate	3	nail	0	15-20°	no	yes	yes	yes	nd	yes
18	**M**	23	EU	F710C	TAD	58	yes	yes	yes	no	>800	yes	severe	1-2	oral	2	<10°	no	no	mild	yes	yes	no
116	**M**	8	EU	T714A	TAD	63	yes	yes	no	yes	>800	yes	severe	>6	oral	nd	10-14°	no	yes	yes	yes	yes	nd
96	**M**	9	EU	g→a ex 22(+1)	TAD	32	no	yes	yes	no	>800	nd	moderate	1-2	no	nd	<10°	no	nd	mild	nd	nd	no
1	**M**	19	EU	no mutation		72	yes	yes	no	yes	>800	yes	severe	>6	nail	>3	10-14°	yes	no	yes	yes	yes	no
4	**M**	17	EU	no mutation		60	yes	no	no	no	>800	no	severe	3	nail	>3	10-14°	yes	yes	yes	yes	yes	yes
9	**M**	30	EU	no mutation		49	yes	no	no	no	700-800	yes	severe	4-6	nail	>3	<10°	no	no	yes	yes	no	no
10	**M**	59	EU	no mutation		29	yes	no	no	no	<700	yes	moderate	3	no	2	<10°	no	no	yes	yes	no	no
11	**F**	55	EU	no mutation		55	yes	yes	yes	no	700-800	no	moderate	>6	nail	0	<10°	yes	no	mild	no	no	no
12	**F**	15	EU	no mutation		38	yes	no	no	nd	700-800	yes	severe	4-6	no	3	<10°	no	no	mild	yes	no	no
14	**F**	34	EU	no mutation		47	yes	yes	no	yes	<700	no	severe	>6	no	0	10-14°	no	no	yes	yes	no	yes
20	**F**	9	EU	no mutation		32	no	yes	no	no	>800	no	mild	1-2	no	3	<10°	no	no	mild	yes	yes	no
21	**M**	8	EU	no mutation	Dock8	49	yes	yes	yes	no	>800	no	severe	3	oral	2	<10°	no	no	mild	yes	nd	no
22	**F**	18	EU	no mutation		43	yes	no	no	no	>800	yes	severe	4-6	nail	>3	<10°	no	no	yes	no	no	no
28	**M**	15	SA	no mutation		44	yes	yes	yes	no	<700	yes	moderate	3	oral	2	<10°	no	yes	no	yes	no	no
35	**M**	7	EU	no mutation		39	yes	yes	yes	no	700-800	nd	severe	nd	nd	0	<10°	no	yes	no	no	yes	no
38	**M**	21	EU	no mutation		57	yes	yes	yes	nd	>800	yes	severe	1-2	no	2	<10°	no	yes	yes	no	nd	no
52	**M**	14	EU	no mutation		35	yes	yes	no	no	>800	no	mild	1-2	no	0	<10°	no	no	mild	no	no	no
54	**M**	21	EU	no mutation		29	no	yes	no	no	>800	no	no	1-2	oral	0	<10°	no	no	no	no	no	no
55	**M**	19	EU	no mutation		26	yes	no	no	no	>800	no	severe	1-2	no	0	15-20°	no	no	no	no	no	no
58	**F**	9	EU	no mutation		38	no	yes	yes	no	<700	yes	moderate	1-2	no	>3	<10°	no	no	mild	nd	no	no
59	**M**	21	EU	no mutation		45	yes	yes	yes	yes	<700	yes	moderate	nd	no	0	10-14°	no	no	nd	no	yes	yes
69	**M**	25	EU	no mutation		61	yes	yes	no	no	>800	yes	severe	4-6	nail	>3	10-14°	yes	no	yes	yes	yes	yes
75	**F**		ME	no mutation		35	yes	yes	yes	yes	>800	nd	moderate	>6	nd	nd	nd	nd	nd	nd	nd	nd	nd
76	**F**	29	ME	no mutation		42	yes	yes	yes	yes	<700	nd	moderate	4-6	nail	0	<10°	no	no	no	no	no	no

78	**M**	6	EU	no mutation		46	no	yes	nd	no	>800	yes	severe	4-6	systemic	nd	<10°	no	no	yes	yes	yes	no
80	**M**	39	ME	no mutation	Dock8	48	yes	yes	yes	no	>800	nd	severe	4-6	systemic	nd	<10°	no	no	no	no	no	no
81	**F**	9	ME	no mutation	Dock8	44	yes	yes	no	yes	>800	nd	moderate	3	nail	0	<10°	no	no	mild	yes	no	no
82	**M**	10	ME	no mutation	Dock8	63	yes	yes	yes	yes	>800	yes	moderate	>6	systemic	3	<10°	no	yes	no	yes	no	no
83	**M**	9	ME	no mutation	Dock8	40	no	yes	yes	yes	>800	nd	moderate	4-6	nail	0	<10°	no	nd	mild	nd	no	no
88	**M**	15	ME	no mutation		53	yes	yes	yes	yes	>800	nd	severe	4-6	no	nd	nd	nd	yes	mild	yes	nd	nd
90	**M**	16	EU	no mutation		29	yes	no	no	no	700-800	no	severe	3	no	2	10-14°	no	no	mild	yes	yes	no
97	**M**	5	EU	no mutation		39	yes	yes	no	yes	>800	yes	severe	1-2	oral	0	nd	no	nd	mild	nd	nd	no
105	**F**	8	EU	no mutation		19	no	no	no	no	<700	no	mild	1-2	no	0	<10°	yes	yes	no	no	no	no
107	**F**		EU	no mutation		26	no	yes	yes	no	<700	no	no	1-2	no	>3	<10°	yes	no	no	no	no	no
112	**M**	4	EU	no mutation		21	yes	no	nd	no	700-800	nd	severe	nd	nd	nd	nd	no	nd	mild	nd	nd	no
113	**F**	5	EU	no mutation		39	no	no	no	yes	>800	yes	severe	1-2	no	0	<10°	no	yes	mild	yes	yes	nd
122	**M**	11	EU	no mutation		29	yes	yes	no	yes	<700	no	moderate	3	no	0	<10°	no	no	no	no	yes	no
123	**F**	24	EU	no mutation		30	yes	no	no	yes	<700	no	severe	1-2	no	2	<10°	no	no	mild	no	no	no
124	**M**	1	EU	no mutation		22	no	no	no	no	>800	no	severe	1-2	nd	0	<10°	no	nd	no	nd	yes	no
127	**F**	7	EU	no mutation		49	yes	yes	yes	no	>800	no	severe	1-2	oral	2	<10°	no	no	mild	yes	yes	yes
137	**M**	11	EU	no mutation		29	no	yes	no	nd	700-800	nd	nd	4-6	nd	3	nd	nd	yes	nd	nd	yes	nd
138	**M**	40	EU	no mutation		43	yes	no	no	no	>800	nd	severe	4-6	no	0	<10°	no	no	yes	yes	no	yes
139	**M**	6	EU	no mutation		40	yes	no	no	yes	>800	no	severe	4-6	oral	nd	<10°	no	yes	mild	yes	yes	no
140	**F**	10	EU	no mutation		38	no	yes	no	no	>800	yes	severe	1-2	no	3	<10°	no	nd	mild	no	yes	no
141	**F**	1	EU	no mutation		30	no	yes	no	yes	>800	no	severe	1-2	no	nd	nd	nd	no	no	no	yes	no
142	**F**	13	EU	no mutation		32	no	no	no	no	>800	yes	moderate	1-2	no	2	10-14°	yes	no	no	no	yes	no
144	**M**	14	EU	no mutation		53	no	yes	yes	no	>800	nd	severe	1-2	no	>3	<10°	no	yes	yes	nd	yes	no
145	**F**	2	EU	no mutation		23	yes	no	no	no	700-800	no	mild	1-2	no	nd	<10°	yes	no	no	no	no	no
146	**F**	20	EU	no mutation		38	no	yes	yes	no	>800	no	moderate	1-2	no	0	<10°	no	yes	no	no	no	no
148	**F**	7	EU	no mutation		35	no	no	no	no	>800	no	severe	>6	no	no	10-14°	no	yes	mild	yes	yes	no
149	**F**	23	EU	no mutation		47	yes	no	no	no	>800	yes	severe	3	nail	3	<10°	no	yes	mild	no	yes	no
150	**F**	16	EU	no mutation		41	no	yes	yes	no	700-800	nd	severe	nd	no	>3	<10°	no	nd	no	no	no	no
155	**F**	4	EU	no mutation		30	no	yes	yes	no	<700	no	mild	1-2	oral	0	<10°	no	yes	no	no	no	no

156	**M**	14	EU	no mutation	50	yes	yes	yes	no	>800	no	severe	3	nail	0	<10°	no	no	mild	yes	no	no
91	F		ME	no mutation		no detailed clinical information available																
161	**F**		EU	no mutation		no detailed clinical information available																
163	F		EU	no mutation		no detailed clinical information available																
165	M		EU	no mutation		no detailed clinical information available																
166	F		EU	no mutation		no detailed clinical information available																
167	F		EU	no mutation		no detailed clinical information available																
168	F		EU	no mutation		no detailed clinical information available																
170	F		EU	no mutation		no detailed clinical information available																
171	F		EU	no mutation		no detailed clinical information available																
172	F		EU	no mutation		no detailed clinical information available																
173	F		EU	no mutation		no detailed clinical information available																
174	M		EU	no mutation		no detailed clinical information available																
176	F		EU	no mutation		no detailed clinical information available																
177	F		EU	no mutation		no detailed clinical information available																
178	F		EU	no mutation		no detailed clinical information available																
179	M		EU	no mutation		no detailed clinical information available																
180	F		EU	no mutation		no detailed clinical information available																
181	F		EU	no mutation		no detailed clinical information available																
182	F		EU	no mutation		no detailed clinical information available																
184	M		EU	no mutation		no detailed clinical information available																

Table Appendix 1: Clinical findings and STAT3 mutations of the 153 analyzed patients´

EU, Europe; F, female; M, male; ME, Middle East; NA, North America; ND, not determined; SA, South America; p.?, unknown effect on protein level.
*Scoring system described in Grimbacher et al. 1999
**Patients having affected family members
Patients with mutations in DOCK8 are shaded in green
The five Sardininan patients who were further studied are shaded in light blue
Patients refered to us without detailed clinical information are shaded in purperl.

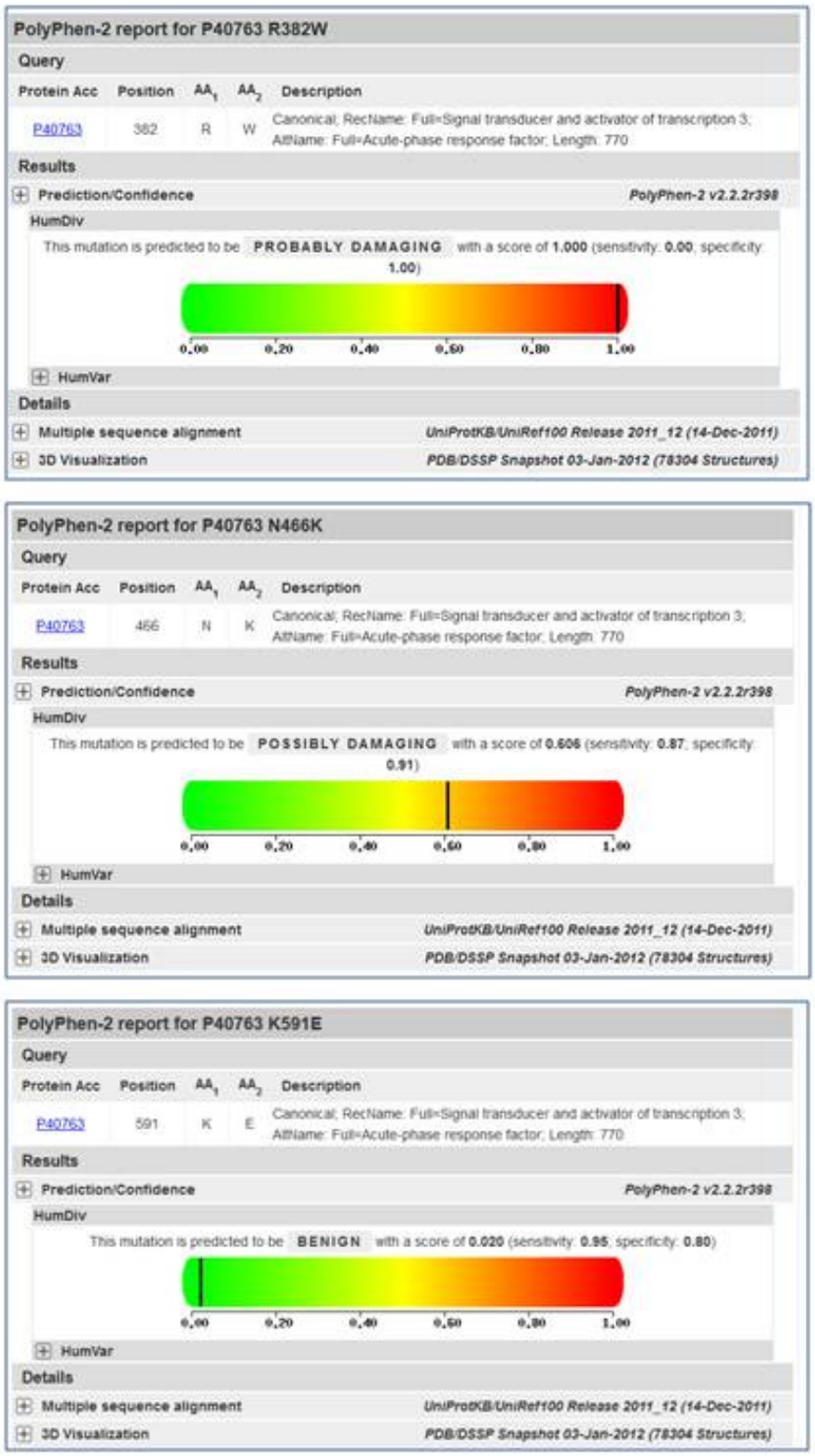

Figure Appendix 1: Polyphen reports

Examples of three Polyphen reports are shown for the three STAT3 mutations R382W, N466K and K591E. The effect of the mutatiob R382W is predicted to be probally damaging, the mutation N466K is classiefied as possibly damaging, K591 E as benign.

Exon	Primer Name		Sequence	Product length (bp)
2	stat3 ex2	fw	5'-ctg tgt tgg gca atg gct act tct-3'	426
		rv	5'-ttg ccc aca tgc cct tct aag ata-3'	
3	stat3 ex3	fw	5'-tga atg ggt tat agc atc agg ttt-3'	364
		rv	5'-tag cca aat gag aaa aga gat gct-3'	
4	stat3 ex4	fw	5'-ctg aag cct ttg ttc cgt tgt ttc-3'	282
		rv	5'-gat ttt cca ttc ctc cca gac cag-3'	
5&6	stat3 ex5&6	fw	5'-caa gcc aaa caa gaa ggg tag tta-3'	883
		rv	5'-ccg cct taa gat cca aac aga g-3'	
7	stat3 ex7B	fw	5'-ctc aaa gga gcc tgg tca tta agg-3'	957
		rv	5'-agt caa ctc cag agc agg aac ttc-3'	
8	stat3 ex7&8	fw	5'-gtt ttt tct tcc ttc gca gac atg-3'	550
		rv	5'-gga aaa gtc ccc acg ttg gag aat-3'	
9&10	stat3 ex9&10B	fw	5'-ttg tag tgg tct cca tgt att cag-3'	595
		rv	5'-aca gtg tac tgc ctg tga cac c-3'	
11	stat3 ex11	fw	5'-cag ctt ggc cta ttt acc tgt tg-3'	310
		rv	5'-agg ctt ttg aaa act ttt gtc cac-3'	
12-14	stat3 ex12-14	fw	5'-tgc gct gat caa ctg taa ctg aat-3'	652
		rv	5'-ggc ctg aag tga ctt ttt gga ata-3'	
15	stat3 ex15B	fw	5'-agc tgg ggc tat agg tgt gc -3'	354
		rv	5'-aaa tcc cag tgg aag ttt ttg gtc -3'	
16	stat3 ex16&17A	fw	5'-ccg tga gat gcg ggt gaa gag att-3'	511
		rv	5'-atg gcc caa atg aac agc cct atg-3'	
18-20	stat3 ex18-20	fw	5'-ctg att att ttg tgg ccc att gtg-3'	867
		rv	5'-ttt tgc gag tct gag tga aac agg-3'	
21	stat3 ex21	fw	5'-agc cag cct gaa tat ttc cac agt-3'	448
		rv	5'-caa gga tcc caa aat ttc caa ctt-3'	
22	stat3 ex22B	fw	5'-aac cct gag ggg cag ctg ga-3'	441
		rv	5'-ggg ggt tga caa gac aca atg g-3'	
23	stat3 ex23B	fw	5'-ccc gtt ttg cct tca ttt cta ata-3'	530
		rv	5'-cac atg tgg cat tca tac cat ctc-3'	
24 coding	stat3 ex24 I	fw	5'-aca gta gat caa ggg cag aag-3'	819
		rv	5'-tct tct att tgg atg tca gca-3'	

Table Appendix 2: Primer table I

Primers used for the amplification of the coding, genomic sequence of STAT3

	Primer Name		Sequence	Product length (bp)
31-647	stat3 mRNA I	fw	5'-gag ggg gag ccg ggg gtt cc-3'	618
		rv	5'-cac ggc tgc tgt ggg gtg gtt g-3'	
368-911	stat3 mRNA 1-2	fw	5'-ggg cat atg cgg cca gca aag aat-3'	544
		rv	5'-agc ccc gcc agc tca ctc acg at-3'	
34-1275	stat3 mRNA II	fw	5'-aat gga gat tgc ccg gat tgt gg-3'	742
		rv	5'-gaa ctg gac gcc ggt ctt gat gac-3'	
896-1510	stat3 mRNA 2-3	fw	5'-gtg agc tgg cgg ggc ttt tgt cag-3'	615
		rv	5'-ctc ggc ccc cat tcc cac atc tct-3'	
1160-1836	stat3 mRNA III	fw	5'-aga gaa tcg tgg agc tgt tta ga-3'	677
		rv	5'-gag ttt ctc tgc cag tgt agt cag-3'	
1603-2289	stat3 mRNA 3-4	fw	5'-gag acc cac tcc ttg cca gtt gtg-3'	687
		rv	5'-gaa tgc ctc ctc ctt ggg aat gtc-3'	
1767-2604	stat3 mRNA IV	fw	5'-ctg gca gtt ctc ctc cac cac caa-3'	838
		rv	5'-tgc agg tag gcg cct cag tcg tat-3'	
2343-2933	stat3 mRNA 4-5	fw	5'-cgc tgc ccc ata cct gaa gac caa-3'	591
		rv	5'-gca ggc acc agg agg cac ttg tc-3'	
2402-3122	stat3 mRNA V	fw	5'-cca ttg acc tgc cga tgtc-3'	721
		rv	5'-gaa agg cta tgc tga tac agt gtt-3'	
2813-3482	stat3 mRNA 5-6	fw	5'-ggg ggt ggc tag agg gag aaa a-3'	670
		rv	5'-gct taa agc acc aag gag gct gtt-3'	
3012-3768	stat3 mRNA VI	fw	5'-cct tgc tga cat cca aat aga aga-3'	757
		rv	5'-taa ggc acc cac aga aac aac cta-3'	
3565-4075	stat3 mRNA 6-7	fw	5'-cct ggc ctt tgg tgt tga aat agg-3'	511
		rv	5'-ttg ccc tgc atg aac tga atg aag-3'	
3649-4488	stat3 mRNA VII	fw	5'-tgt aat gta ttg gcc ttt tag tga-3'	-3'840
		rv	5'-aga gtg ttg ctg gag aag taa gag-3'	
4034-4724	stat3 mRNA 7-8	fw	5'-tgg cac ttg taa tgg cgt ctt cat-3'	691
		rv	5'-ggg cct gag gac cct gtt ctt ta-3'	
4356-4950	stat3 mRNA VIII	fw	5'-tta aat caa att agc tgg tct ctg-3'	595
		rv	5'-tct gga agt taa agt aga tac agc-3'	

Table Appendix 3: Primer table II

Primers used for the amplification of the cDNA of the Signal Transducer and Activator of Transcription STAT3 (transcript NM_139276.2)

Exon	Primer Name		Sequence	Product length (bp)
1	IL6 exon 1	fw	5'-agg cgg gtc ctg aaa tgt tat gc-3'	624
		rv	5'-ctc tcc cca ttg cca ctg agt ctc-3'	
2&3	IL6 exon 2&3	fw	5'-aat cag ccc cac ccg ctc tgg-3'	702
		rv	5'-gcc ccg ttg gcc tca aat cta cag-3'	
4	IL6 exon 4	fw	5'-tgc ccc cac tcc act gg-3'	428
		rv	5'-gct tga agt tgg cct cct cat-3'	
5	IL6 exon 5	fw	5'-tgc ccc cac tcc act gg-3'	428
		rv	5'-gct tga agt tgg cct cct cat-3'	
6	IL6 exon 6	fw	5'-gca ccc cag tta atc tca ttc a-3'	781
		rv	5'-gtg gtg gca gtg aca aga aac t-3'	

Table Appendix 4: Primer table III

Primers used for the amplification of the coding, genomic sequence of IL-6

	Primer Name		Sequence	Product length (bp)
4-738	IL-6RAmRNA 1	fw	5'-ggt ccc ctg ttc tcc ccg ctc ag-3'	735
		rv	5'-ggc cgg ccc ggt agc atg aat agt-3'	
569-1334	IL-6RAmRNA 2	fw	5'-tct gac ctg ccc ggg ggt aga gc-3'	766
		rv	5'-ccc gaa ctc ctc ctg ggc acg aa-3'	
1150-1954	IL-6RAmRNA 3	fw	5'-ggc aag acc ccc act cct gga act-3'	805
		rv	5'-agg ccg cac acc cct gag ata agc-3'	
1794-2579	IL-6RAmRNA 4	fw	5'-cca cgg agc cct tat gac atc agc-3'	786
		rv	5'-caa agc caa tgc agt tgc ctc tgt-3'	
2352-3127	IL-6RAmRNA 5	fw	5'-gcc tgg gga cgg ctt tta ctt-3'	776
		rv	5'-att gcc cag gct gat ctc aaa ctt-3'	
2965-3642	IL-6RAmRNA 6	fw	5'-gtg tgg ggg aag cac cat aac t-3'	678
		rv	5'-aaa aga gga agg cac cga ggt aag-3'	
3437-4176	IL-6RAmRNA 7	fw	5'-gaa aaa cca gcg tgt gac tac tcc-3'	
		rv	5'-ccc agg ttg gtc tca aac tcc-3'	

Table Appendix 5: Primer table IV

Primers used for the amplification of the cDNA of the IL-6 receptor A (IL-6RA) (transcript NM_139276.2)

	Primer Name		Sequence	Product length (bp)
75-812	gp130mRNA 1	fw	5'-ctg cca ggc cga cgg gtc-3'	738
		rv	5'-gtg cat gag gtg ggg gtg tca cg-3'	
675-1500	gp130mRNA 2	fw	5'-cga ggg gaa gaa aat gag gtg tga-3'	826
		rv	5'-agc tgc atc tga ttt gcc aac aa-3'	
1324-2067	gp130mRNA 3	fw	5'-ttg cct cct ttt gaa gcc aat g-3'	744
		rv	5'-ctt ccc acc ttc atc tgt gta tgc-3'	
1952-2785	gp130mRNA 4	fw	5'-gaa atg aaa ctg ctg tga atg tgg-3'	834
		rv	5'-tga ctg atg aaa ctt gct ttg acc-3'	
2592-3397	gp130mRNA 5	fw	5'-ctt ctc aag atc cga gtc tac c-3'	806
		rv	5'-caa aat cag tat agc tgt gct cac-3'	
3248-4147	gp130mRNA 6	fw	5'-gat ttt gtg ctg ttt cag gat gtt-3'	900
		rv	5'-caa gtt gtc tta gtg tgc ctc aaa-3'	
4001-4797	gp130mRNA 7	fw	5'-tca gag tac tgg gta gat gaa c-3'	797
		rv	5'-ttt gcc taa agt ggg aaa ct-3'	
4637-5461	gp130mRNA 8	fw	5'-tct cag aat att gtt cac att aga-3'	825
		rv	5'-cta gtt ttt aca taa ata acc aga-3'	
5292-6052	gp130mRNA 9	fw	5'-att tga gca cta ata tgt aat gt-3'	761
		rv	5'-cta ttt ctc aaa cta gat gct c-3'	
5905-6684	gp130mRNA 10	fw	5'-aag gga ttt tac cac atg aag tc-3'a	780
		rv	5'-tag gtt aat gca aaa gat aat acg-3'	
6515-7330	gp130mRNA 11	fw	5'-cat tgt cat tga ttt caa aga agg-3'	816
		rv	5'-gca att cac tgc aaa ggt aaa-3'	
7169-8072	gp130mRNA 12	fw	5'-tga cta gca tta ggt atg ttt ctt-3'	904
		rv	5'-tat atg tga agg agt tac tgt tgg-3'	
7888-8438	gp130mRNA 13	fw	5'-cat ggc atc ctc ctg cta ctt ctt-3'	551
		rv	5'-taa ctt gcc caa gca ctg gac tta-3'	
8269-8964	gp130mRNA 14	fw	5'-ttt ctc tag ccc aaa ggc agt g-3'	696
		rv	5'-acc ata tag ctc acc atg tta tcc-3'	

Table Appendix 6: Primer table V

Primers used for the amplification of the cDNA of the IL-6 receptor signalling unit gp130 (transcript NM_139276.2)

	Primer Name		Sequence	Product length (bp)
20-635	Jak1 mRNA 1	fw	5'-cgc cca gtc ccg gct gtc ctc-3'	616
		rv	5'-tga cgc cac act gac tgc tca ttg	
565-1283	Jak1 mRNA 2	fw	5'-cac tac cgg atg agg ttc tat ttc-3'	719
		rv	5'-ttt ttc cgc ttc agt tta ttt tt-3'	
1166-1789	Jak1 mRNA 3	fw	5'-acg gtg gaa acg ttc tct act acg-3'	624
		rv	5'-ggt ccg aac cgt gca gac tgt-3'	
1534-2161	Jak1 mRNA 4	fw	5'-ccc ccg ttg atc gtc cac a-3'	628
		rv	5'-cct cga aga agg cca ggg aaa ta-3'	
2044-2757	Jak1 mRNA 5	fw	5'-tct ggg acc ctg atg gat tac aag-3'	714
		rv	5'-agg cct ctg att ggg gtc ata gtt-3'	
2590-3244	Jak1 mRNA 6	fw	5'-acc acg ctc tgg gaa atc tgc tac-3'	655
		rv	5'-att gcc gag aac cca aat agt cca-3'	
3083-3836	Jak1 mRNA 7	fw	5'-cag aag acg gag gaa atg gta tta-3'	754
		rv	5'-ttc agt gac ttt ttg gac aga aca-3'	
3636-4430	Jak1 mRNA 8	fw	5'-gaa atg ctg gga att cca acc at-3'	795
		rv	5'-ctg gca cag gct tag ttc ttg aga-3'	
4162-5000	Jak1 mRNA 9	fw	5'-gac tta gcc ctc aaa ttt cag tat-3'	839
		rv	5'-ata gag tgg cca cag gtt tga cac	

Table Appendix 7: Primer table VI

Primers used for the amplification of the cDNA of the receptor associated tyrosine kinase JAK1 (transcript NM_139276.2)